Establishment of a model system for studying polyacetylene biosynthesis in Asteraceae and studies on transformation and cryopreservation of carrot cells

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Abbreviations

2,4-D	2,4-Dichlorophenoxyacetic acid
ACP	Acyl carrier protein
AM4/1	Modified Murashige and Skoog medium
AP	Alkalin phosphatase
BAP	Benzylaminopurin
BSA	Bovine serum albumin
CAPS	N-cyclohexyl-3-aminopropanesulfonic acid
cDNA	Complementary deoxyribonucleic acid
CoA	Coenzyme A
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTPs	Deoxynucleotide triphosphates
DSMZ	*Deutsche Sammlung von Mikroorganismen und Zellkulturen*
DTT	1,4-Dithiothreitol
DW	Dry weight
E.coli	*Escherichia coli*
EDTA	Ethylenediaminetetraacetic acid
FAD	Fatty acid desaturase
FDU	Flourodesoxyuridine
GC-FID	GC with flame ionization detection
GC-MS	Gas chromatography-mass spectrometry
IAA	Indole-3-acetic acid
IBA	Indole-3-butyric acid
LB	Lysogeny broth
m/z	Mass per charge
MW	Molecular weight
NAA	1-Naphthaleneacetic acid
OD_{600nm}	Optical density at $\lambda = 600$ nm
ORF	Open reading frame
PAGE	Polyacrylamide gel electrophoresis
PCR	Polymerase chain reaction
RI	Retention index

SD	Standard deviation
SDS	Sodium dodecyl sulfate
SOC	Super optimal broth medium with addition of glucose
TBE-buffer	Tris-borate-EDTA-buffer
TBS	Tris-buffered saline
Tm	Melting temperature
Tris	Tris(hydroxymethyl)aminomethane
TTBS	Tween 20-tris-buffered saline
USER	Uracil-specific excision reagent
UV	Ultraviolet
YEP	Yest extract peptone
YMA	Yeast mannitol agar
YMB	Yeast mannitol broth

1 Introduction

1.1 Specialized metabolites in plant tissue culture

The term plant tissue culture refers to growing and multiplication of cells, tissues and organs of plants on defined artificial solid or liquid media under aseptic, controlled conditions (Thorpe, 2007). It applies to various types of plants ranging from food crops to medicinal plants and trees. The theoretical basis for plant tissue culture was proposed by the German physiologist Gottlieb Haberlandt in 1902. He isolated single palisade cells from leaves in a salt solution supplemented with sucrose. Through one month, the cells increased in size and accumulated starch, but failed to divide. Despite the failure, Haberlandt is considered the godfather of plant tissue culture (Haberlandt, 1902). Today, tissue culture is widely used in plant breeding, micropropagation of crop plants, and plant research. It is fundamental for genetic transformation of most plant species as transformation methods mostly rely on regeneration of plants from transformed cells or tissue cultures (Hardegger and Sturm, 1998; Franklin et al., 2007; Iantcheva et al., 2014). Commercial production of therapeutic proteins in plant tissue culture such as glucocerebrosidase for treatment of Gaucher's disease indicates its high potential as pharmaceutical production platforms (Shaaltiel et al., 2007; Kaiser, 2008; http://www.greenovation.com/; searched on 17 March 2016).

As specialized (or "secondary") metabolism is not essential for growth and development of a plant but mediates interactions of a plant with its environment, plant tissue cultures may or may not produce specialized metabolites (Hussain et al., 2012). Organ cultures like root cultures often produce the same special metabolites as the intact plants (Margl et al., 2002). Root cultures are therefore often used as a model system for biochemical studies of biosynthetic pathways of specialized metabolites. However, over several passages biosynthesis of specialized metabolites might be downregulated as there is no need for the cells to produce e.g. defense compounds in a sterile environment. Un-organized cultures such as cell suspension or callus cultures often produce only small amounts of specialized metabolites or none (Wink, 1993). Although plant tissue cultures have been proposed as a source of high-value specialized metabolites (Alfermann and Petersen, 1995; Scragg, 1997), their commercial success has been limited due to the difficulty to stably produce sufficient amounts of the desired compounds (DiCosmo and Misawa, 1995). In some cases, elicitors such as methyl jasmonate, chitosan and heavy metals can be used to induce signalling pathways that might lead to increased specialized metabolite production (Poulev et al., 2003; Bota and Deliu, 2011). With respect to structurally complex specialized metabolites, the production in plant tissue culture

is also complicated by the need to include several different or specialized cell types which contribute to the biosynthetic pathway in the intact plant (St-Pierre et al., 1999; Weid et al., 2004). However, examples of successful commercial production of specialized metabolites by means of plant tissue culture are the production of shikonin in cell suspension cultures of *Lithospermum erythrorhizon* (Boraginaceae) (Fukui et al., 1983), of berberine in cell suspension cultures of *Coptis japonica* (Berberidaceae) (Sato and Yamada, 1984; Morimoto et al., 1988), and of paclitaxel in cell suspension cultures of *Taxus brevifolia* (Taxaceae) (http://www.phytonbiotech.com/paclitaxel/; searched on 12 April 2016). One advantage of cell suspension cultures in this context lies in the possibility of a scale-up for industrial fermentation (http://www.phytonbiotech.com/paclitaxel/; searched on 12 April 2016). Furthermore, specialized metabolites can be obtained under standardized conditions without the need to grow plants in the field or to collect plant material from natural resources.

Despite the limitations for commercial production of specialized metabolites, plant tissue culture has been widely used to study biosynthetic pathways of specialized metabolites (Gaosheng and Jingming, 2012). Tissue culture offers the advantage that experiments to study biosynthesis can be conducted under controlled, reproducible conditions, with little space requirements, with relatively short growth periods and independently of growth facilities for intact plants. Elicitors can easily be added to achieve high biosynthetic rates in the induced cultures as compared to untreated controls.

With the advancement of genetic engineering techniques and the elucidation of an increasing number of biosynthetic pathways of plant specialized metabolites, plant tissue cultures, especially cell suspension cultures, have become an interesting host for reconstitution of a complete biosynthetic pathway for a compound of interest by heterologous expression of several biosynthetic genes. In this way, biosynthetic enzymes would be expressed in the environment of a plant cell (as opposed to hosts such as yeast or bacteria) which might be advantageous for e.g. plastidial enzymes. When compared to production of endogenous specialized metabolites by untransformed cell suspension cultures, such engineered cultures offer the possibility to adjust expression levels and to avoid gene silencing through the choice of promoters driving expression of the transgenes.

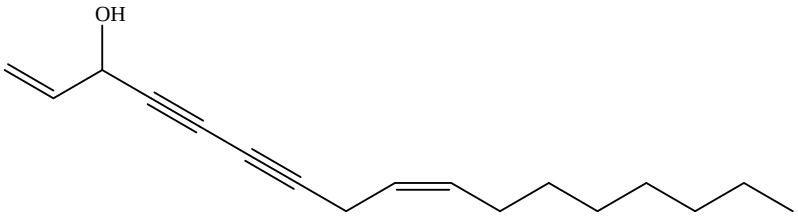
The present thesis was aimed at exploring the potential of plant tissue culture in the study of a group of plant specialized metabolites known as polyacetylenes whose biosynthesis is poorly understood. Furthermore, the well characterized biosynthetic pathway of another group of compounds, the glucosinolates, was used to start evaluating transgenic plant cell suspension cultures as production platform for specialized metabolites.

1.2 Polyacetylenes in plants

1.2.1 Definition, properties and biological activity of polyacetylenes

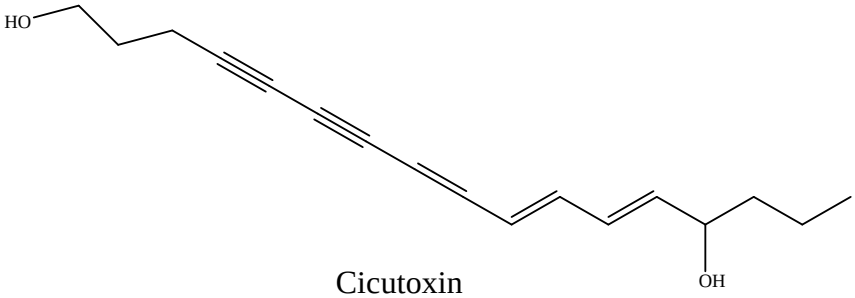
Polyacetylenes or polyynes are unsaturated, bioactive specialized metabolites that contain not only double bonds but also C-C triple bonds (Fig. 1.1) (Kaufman et al., 1998; Minto and Blacklock, 2008). Compounds derived from a polyacetylenic precursor but lacking the triple bond are also commonly included in this group of compounds. Polyacetylenes are often unstable and readily undergo changes in their chemical structure. They are very sensitive towards heat and light and sometimes damaged during distillation (Schrader et al., 2005) . This instability often leads to difficulties in their isolation, quantification and characterization (Bohlmann et al., 1973; Christensen and Brandt, 2006). Most polyacetylenes are lipophilic. Lipophilicity decreases with decreasing chain length and presence of hydrophilic groups. Polyacetylenes are widespread in nature. They occur in plants, fungi (Bohlmann et al., 1973), algae (De Napoli et al., 1981), and sponges (Seo et al., 1998; Kim et al., 2002). In plants, the polyacetylenes are found in several families but are especially widely distributed in the Asteraceae, Apiaceae and Araliaceae (Kaufman et al., 1998).

Polyacetylenes can generally be found in above- and below-ground organs of plants. The concentration and distribution of polyacetylenes in plants is quite variable and depends on the season (Hudson, 1989). Some plants do not normally produce polyacetylenes unless they are attacked by a pathogen. For example, fruits and leaves of tomato *Solanum lycopersicum* (Solanaceae) produce falcarinol and falcarindiol only when infected with leaf mould *Cladosporium fulvum* (De Wit and Kodde, 1981).



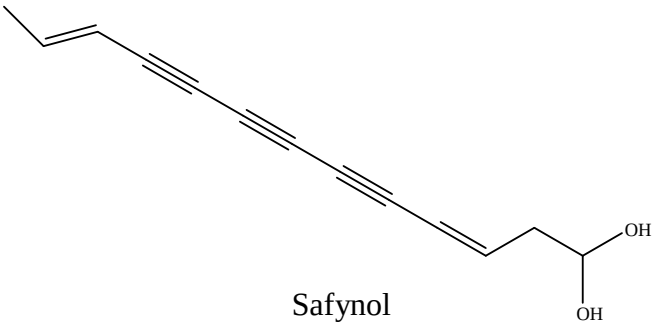
Falcarinol

Daucus carota
commons.wikimedia.org



Cicutoxin

Cicuta virosa
commons.wikimedia.org



Safynol

Carthamus tinctorius
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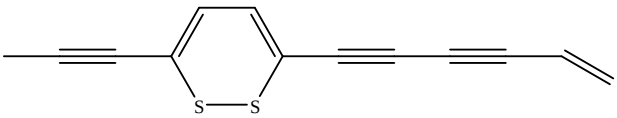

Thiarubrine A

Ambrosia artemisiifolia
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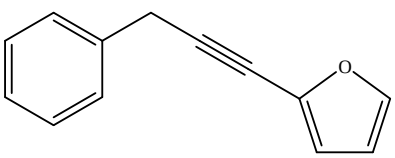


Carlinaoxid

Carlina acaulis
commons.wikimedia.org

Fig. 1.1: Examples of polyacetylene structures. Apiaceae and Araliaceae are characterized by C_{17}-aliphatic polyacetylenes. Falcarinol is present in edible vegetables such as carrot (*Daucus carota*). Cicutoxin found in Water hemlock (*Cicuta virosa*) is highly toxic to mammals. Asteraceae are characterized by a diversity of aliphatic polyacetylenes such as safynol, a phytoalexin found in *Carthamus tinctorius,* and aromatic polyacetylenes such as thiarubrine A found in *e.g. Ambrosia artemisiifolia.* Carlinaxoid is an antibacterial polyacetylene found in *Carlina acaulis.*

The structural types of polyacetylenes differ between the closely related families Apiaceae and Araliaceae and the Asteraceae family (Fig. 1.1). While C_{17}-4,6 diynes with a variable number of additional double bonds and hydroxyl functions dominate in the Apiaceae and Araliaceae, Asteraceae accumulate structurally diverse polyacetylenes such as thiarubrines, furans, pyrans, tetrahydropyrans, tetrahydrofurans, and aromatic as well as aliphatic polyacetylenes.

The biological properties of polyacetylenes have attracted the attention of many plant pathologists and pharmacologists. As shown in Tab. 1.1, polyacetylenes have antifungal, antibacterial, antiviral, insecticidal, anti-inflammatory, and antitumor activity. Medicinal use of pure polyacetylenes is limited by their chemical instability. However, polyacetylenes are present in vegetable foods such as carrot, celery and parsley and might contribute to health effects associated with the consumption of these vegetables. However, some polyacetylenes are undesirable in plant foods due to their toxicity. For example, cicutoxin from the Water hemlock (*Cicuta virosa*; Apiaceae) and oenanthotoxin from Hemlock water dropwort (*Oenanthe crocata*; Apiaceae) are very toxic to mammals. Ingestion of these plants leads to vomiting, convulsions, respiratory paralysis and death (Panter et al., 1988; Panter et al., 2011). Ichthythereol and its acetate from *Ichthyothere terminalis* (Asteraceae) also shows neurotoxic effect and is widely used by native South American indians as a fish poisoning (Cascon et al., 1965; Chin et al., 1965).

Tab. 1.1: Biological activity of different polyacetylenes from the families Apiaceae, Asteraceae and Araliaceae.

Effect	Family	Substance	Source	Reference
Antibacterial	Asteraceae	Carlinaoxid	*Carlina acaulis*	(Bohlmann and Mannhardt, 1957)
		Safynol	*Carthamus tinctorius*	(Bohlmann et al., 1966)
Neurotoxic	Apiaceae	Cicutoxin	*Cicuta virosa*	(Anet et al., 1953)
		Oenanthotoxin	*Oenanthe crocata*	(Anet et al., 1953)
	Asteraceae	Ichthythereol	*Ichthyothere terminalis*	(Chin et al., 1965)
Cytotoxic	Araliaceae	Panaxacol	*Panax ginseng*	(Fujimoto and Satoh, 1987)
		Panaxydol	*Panax ginseng*	(Ahn and Kim, 1988)
		Panaxytriol		
Antiviral	Asteraceae	α-Terthienyl	Tagetes-species	(Hudson, 1989)
		Thiarubrine A		
		Phenylheptatriyne		
Antifungal	Apiaceae, Araliaceae	Falcarinol	*Daucus carota*, *Panax ginseng*	(Olsson and Svensson, 1996) (Hansen and Boll, 1986)
		Falcarindiol		
Insecticidal-larvicidal	Asteraceae	α-Terthienyl	Tagetes-species	(Arnason et al., 1981)
		Thiarubrine A		
		Phenylheptatriyne		
Antiinflamatory	Asteraceae	En-yn-dicycloether	*Chamomilla recutita*	(Murti et al., 2012)
		Safynol-2-O-isobutyrate	*Bidens campylotheca*	(Redl et al., 1994)

Among polyacetylenes, especially thiophenes and dithiin derivatives are phototoxic under UVA light (320-400 nm) for a wide range of organisms, including nematodes, fungi, bacteria,

viruses, and mammalian cells (Hudson, 1989; Hudson and Towers, 1991; Kagan et al., 1992). For example, nematocidal activity of thiarubrine A and related compounds is enhanced by light (Towers et al., 1985). Thiarubrine A is also highly phototoxic to membrane-containing viruses but only slightly phototoxic to viruses without membrane (Hudson, 1989; Hudson and Towers, 1991). Upon exposure to ultraviolet (UV) or visible light, thiarubrines are converted to bicyclic photosulfide intermediates that yield thiophenes via desulfurization and free sulfur forming cyclooctasulfur (S_8) (Fig. 1.2) (Page et al., 1999). Thus, phototoxicity of thiarubrine A could be an effect of the compound itself, the derived thiophene or the process of photo-conversion (Page et al., 1999).

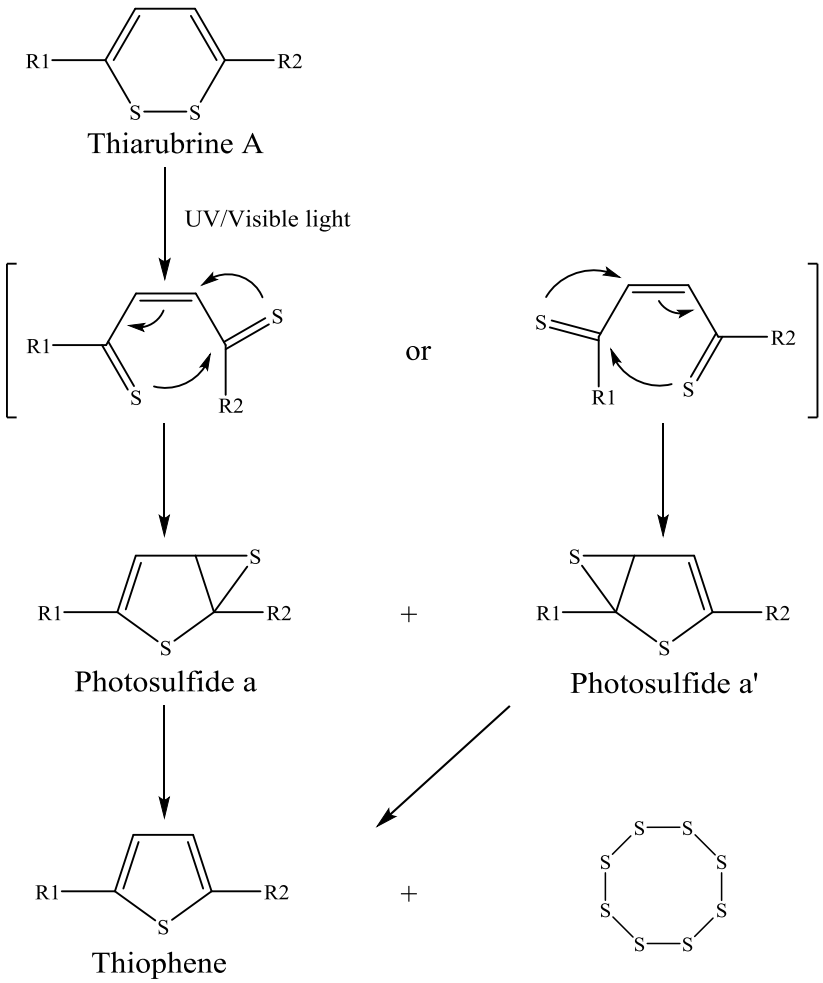


Fig. 1.2: Proposed photochemical conversion of thiarubrines to thiophenes. The photo-conversion of thiarubrines to thiophenes resulted in the formation of photosulfides that upon desulfuration lead to the formation of thiophene with release of cyclooctasulfur. Redrawn from Page et al., (1999). For thiarubrine A: R1 = $H_3C{-}C{\equiv}C{-}$, R2= $H_2C{=}CH{-}C{\equiv}C{-}C{\equiv}C{-}$.

1.2.2 Biosynthesis of polyacetylenes

The similarity of polyacetylene structures with oleic and linoleic acid suggest that polyaceylenes are biosynthesized from unsaturated fatty acids. Feeding experiments with ^{14}C- and ^{3}H-labeled precursors have proved this assumption (Bohlmann et al., 1973; Minto and Blacklock, 2008). As shown in Fig. 1.3, the first step in the biosynthesis of polyacetylenes is the dehydrogenation of linoleic acid to crepenynic acid (18:2 (9c, 12a)), a fatty acid with a triple bond in position 12 (Minto and Blacklock, 2008). Crepenynic acid is considered a branching point between primary and secondary metabolism because it is found as component of lipids in some plants, but also is the first known metabolite on the pathway to acetylenic specialized metabolites. For example, the seed oil of *Crepis alpina* (Asteraceae) contains up to 70 % of crepenynic acid but the plant has not been described to contain polyacetylenes (Bohlmann et al., 1973). In the polyacetylene biosynthetic pathway crepenynic acid is desaturated at C_{14} to form dehydrocrepenynic acid. After that, α- and β-oxidation as well as other oxidative degradation reactions form polyacetylene precursors of various chain lengths, which are transformed into a large diversity of polyacetylene structures.

Only few enzymes of polyacetylene biosynthesis have been identified and characterized. The first enzyme identified in the pathway was Crep1, a Δ^{12}-acetylenase from *C. alpina*, a homolog of FAD2, Δ^{12}-oleic acid desaturase (Lee et al., 1998). FAD2 and Crep1 are membrane bound enzymes which require a reductase partner and NADH as electron donor (Shanklin and Cahoon, 1998). The substrates of both enzymes are not the free fatty acids but membrane-bound fatty acids. Crep1 catalyzes the conversion of linoleic acid to crepenynic acid, but can also act as Δ^{12}-desaturase with oleic acid as substrate (Carlsson et al., 2004). The full-length cDNA of Crep1 from *C. alpina* encodes a 375 amino acid protein having 56 % amino acid sequence identity with *Arabidopsis thaliana* (Brassicaceae) FAD2 (Lee et al., 1998). The Crep1 gene was expressed in *Saccharomyces cerevisiae*. As *S. cerevisiae* does not produce polyunsaturated fatty acids, the cultures were fed with linoleic acid. This allowed the production of crepenynic acid to 0.3 % of total fatty acid content of the yeast (Lee et al., 1998). In addition to the introduction of an acetylenic bond at C_{12} of linoleic acid, Crep1 is also able to introduce a *cis* or *trans* double bond at the C_{12} position of oleic acid. Thus, Crep1 has a bifunctional Δ^{12}-desaturase/Δ^{12}-acetylenase activity (Carlsson et al., 2004). A Crep1 homolog with Δ^{12}- acetylenase activity designated as ELI12 has also been identified in *Petroselinum crispum* (parsley, Apiaceae) (Kirsch et al., 1997; Cahoon et al., 2003). *ELI12* was only expressed in parsley suspension cultures when they were induced by the fungal oligopeptide elicitor Pep25 (Kirsch et al., 1997). Expression of *P. crispum ELI12* in developing soy-

bean seed embryos led to the formation of crepenynic acid and dehydrocrepenynic acid from endogenous linoleic acid (Cahoon et al., 2003). Further studies showed that *Crep1*/*ELI12* homologs are also expressed in sunflower (*Helianthus annuus*, Asteraceae), Calendula (*Calendula officinalis,* Asteraceae) and English ivy (*Hedera helix*, Araliaceae) (Cahoon et al., 2003). Expression of the *ELI12* homologs from all three species in soybean somatic embryos led to accumulation of crepenynic and dehydrocrepenynic acids (Cahoon et al., 2003). Crep1/ELI12 homologs introduce a triple bond with a distance of one methylene group to the previous double bond similar to fatty acid desaturases. Polyacetylenes are, however characterized by conjugated double and triple bonds. No enzyme catalyzing such a reaction in polyacetylene biosynthesis has been identified to date. Acetylenases have also been identified from other organisms. For example, a bifunctional Δ^6-acetylenase/desaturase has been isolated from the moss *Ceratodon purpureus* (Ditrichaceae) (Sperling et al., 2000), a Δ^{12}-acetylenase from the fungus *Cantharellus formosus* (Cantharellaceae) (Blacklock et al., 2010).

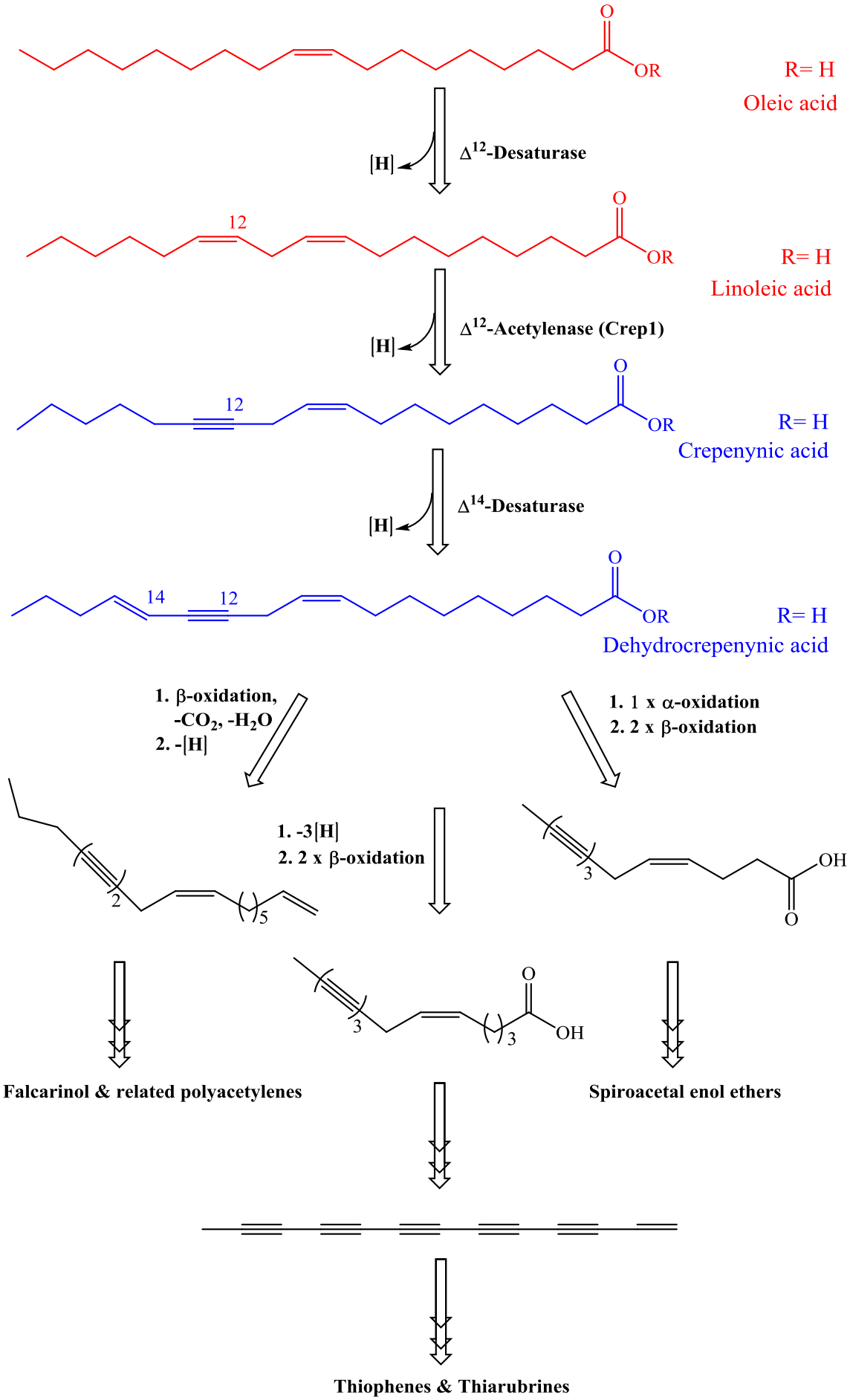

O
OR
R= H
Oleic acid
[H]
Δ12-Desaturase
12
O
OR
R= H
Linoleic acid
[H]
Δ12-Acetylenase (Crep1)
12
O
OR
R= H
Crepenynic acid
[H]
Δ14-Desaturase
14
12
O
OR
R= H
Dehydrocrepenynic acid
1. β-oxidation,
-CO2, -H2O
2. -[H]
1. 1 x α-oxidation
2. 2 x β-oxidation
1. -3[H]
2. 2 x β-oxidation
2
5
3
OH
O
3
3
O
OH
Falcarinol & related polyacetylenes
Spiroacetal enol ethers
Thiophenes & Thiarubrines

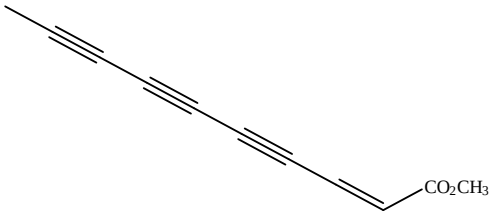
Fig. 1.3: The proposed scheme for the biosynthesis of polyacetylenes redrawn with modification from Minto and Blacklock (2008). No enzyme downstream of Crep1 has been identified yet. Chain shortening, presumably by α- and β-oxidation, forms polyacetylene precursor of various chain lengths. The fatty acid substrates are not free but esterified (R=ACP, CoA or lipid). Red: Primary metabolism; blue: Interface between primary and specialized metabolism; black: Specialized metabolism.

1.2.3 Polyacetylenes in Asteraceae

With about 20,000 species, the Asteraceae constitutes one of the largest plant families forming approximately 10 % of the flowering plants (Herout, 1971). Species of this family have large economic benefits as vegetables (artichokes, lettuce), sources of oil (sunflower, safflower), and garden ornamentals (marigold, chrysanthemum and others). In species of the Asteraceae, more than 1100 different polyacetylenes have been identified, more than half of the polyacetylenes known from higher plants (Minto and Blacklock, 2008). The structural types of polyacetylenes (aliphatic, aromatic, dicycloether, S-containing, and others) are specifically distributed in different tribes of the Asteraceae (Minto and Blacklock, 2008).

Among the aliphatic polyacetylenes those with C_{10}-, C_{13}-, C_{14}-, and C_{17}-chain lengths and with ene-diyne-diene, ene-diyne-ene, diyne-ene, triyne-diene, triyne-ene chromophores are the most commonly found ones (Fig. 1.4). Compounds with four or five triple bonds in conjugation are also widespread in Asteraceae (Binder et al., 1990a). In 1826, the C_{10}-polyacetylene dehydromatricaria ester from Artemisia species was isolated as the first naturally acetylenic compound from plants as reported by Bohlmann et al. (1973). This compound is found in Goldenrod (*Solidago altissima*) (Kawazu et al., 1977) and Wormwood (*Artemisia absinthium*) (Greger, 1978). It has been shown that this compound has an ovicidal activity against the fruit-fly, *Drosophila melanogaster* (Kawazu et al., 1977). 1-Tridecene-3,5,7,9,11-pentayne isolated from the root of *A. lappa* is the most widespread C_{13}-polyacetylenes found in Asteraceae (Takasugi et al., 1987). It is considered a precursor of thiophenes (Arroo et al., 1995). Safynol and dehydrosafynol (Fig. 1.4) are C_{13}-polyacetylenic diols that have been isolated from safflower (*C. tinctorius*) infected with *Phytophthora drechsleri or P. megasperma* (Allen and Thomas, 1971a). Although both compounds have antifungal activity, dehydrosafynol is more fungitoxic than safynol (Allen and Thomas, 1971a). This has been attributed to the higher number of acetylenic bonds. However, safynol has been found to be more stable than dehydrosafynol (Allen and Thomas, 1971a). In addition to the antifungal activity, safynol has also antibacterial activity (Bohlmann et al., 1966). Artemisia ketone (Fig. 1.4) is a C_{14}-polyacetylene found as a principal component of the seed oil from *Artemisia annua* (Woerdenbag et al., 1993). It has antibacterial activity against gram-positive and gram-negative bacteria (Ćavar et al., 2012). Dehydrofacarinol and dehydrofalcarindiol (Fig. 1.4) are

C_{17}-polyacetylenes that have been isolated from various Asteraceae (Christensen and Lam, 1991a; Christensen and Lam, 1991b; Christensen, 1992). They are present in the edible part of Terragon (*Artemisia dracunculus*) (Jakupovic et al., 1991) and Jerusalem artichoke (*Helianthus tuberosus*) (Bohlmann et al., 1962). These compounds are cytotoxic to tumor cell lines with dehydrofalcarinol being more effective than dehydrofalcarindiol (Bernart et al., 1996).



Dehydromatricaria ester

Artemisia absinthium
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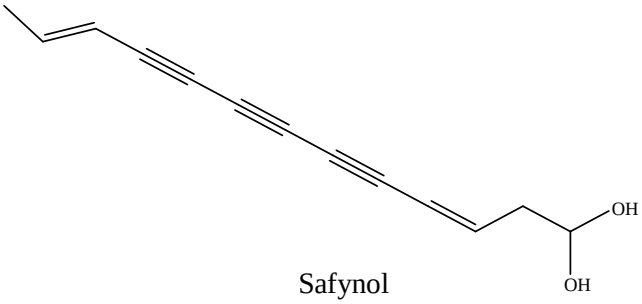

Safynol

Carthamus tinctorius
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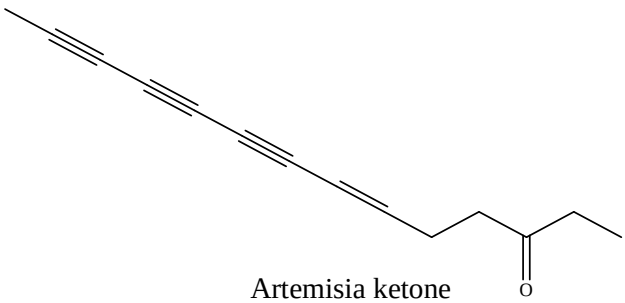

Artemisia ketone

Artemisia annua
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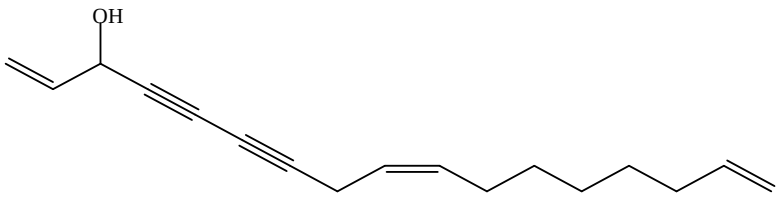

Dehydrofalcarinol

Artemisia absinthium
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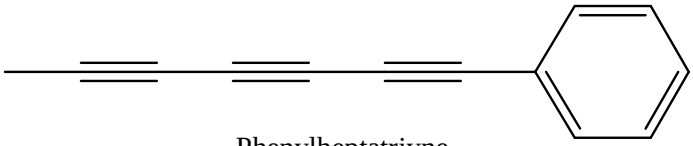
Fig. 1.4: Examples of aliphatic polyacetylenes in Asteraceae.

The majority of the aliphatic polyacetylenes in Asteraceae has a high number of conjugated C-C triple and double bonds. Besides aliphatic polyacetylenes, Asteraceae are also charac-

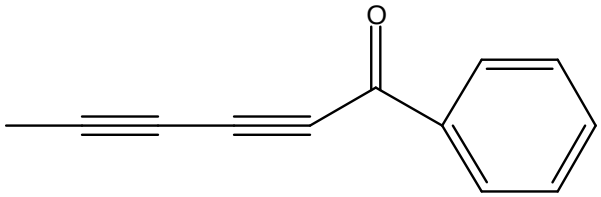
terized by aromatic polyacetylenes. Phenylheptatriyne (Fig. 1.5) is an aromatic polyacetylene found in *Bidens pilosa* (Wat et al., 1979) and *Bidens alba* (Cantonwine and Downum, 2001). It has antimicrobial activity in the presence of UVA (320-400 nm) (Wat et al., 1979) and is an insect antifeedant (McLachlan et al., 1982). Capillin (Fig. 1.5) is an aromatic polyacetylenes from *Artemisia capillaris* (Yano, 1983) and *A. monosperma* (Whelan and Ryan, 2004) that has antitumor (Whelan and Ryan, 2004), anti-inflamatory (Joshi, 2013), and antifungal activities (Christensen, 1998). The cis and trans en-yn-dicycloether (Fig. 1.5) found in the flowers of German chamomile (*Matricaria recutita)* (Buono-Core et al., 2011) has anti-inflammatory, antispasmodic and antifungal activities (Achterrath-Tuckermann et al., 1980; Sharafzadeh and Alizadeh, 2011).

Sulfur-containing polyacetylenes are also widely distributed within Asteraceae (Bohlmann et al., 1973; Christensen and Lam, 1990; Christensen and Lam, 1991b; Christensen, 1992). The most common polyacetylenic sulfur compounds in the Asteraceae are monothiophenes. Most dithiophenes and all trithiophenes do not possess triple bonds, but are biosynthesized from polyacetylenic precursors. The thiophenes of Tagetes species like *T. patula* (Margl et al., 2002) have phototoxic activity against nematodes (Gommers, 1972), herbivorous insects (Champagne et al., 1986), viruses (Hudson et al., 1986) and fungi (Mares et al., 1990) (see chapter 1.2.4). Cyclic dithiynes such as thiarubrine A (Fig. 1.5) are another characteristic group of sulfur containing polyacetylenes in the Asteraceae. They accumulate to high levels in *Ambrosia artemisiifolia* (Bhagwath and Hjortsø, 2000) and *A. maritima* (Zid and Orihara, 2005). They are characterized by an intense wine-red color and are toxic to many organisms, e.g. bacteria (Towers et al., 1985), virus (Hudson, 1989), and fungi (Ellis et al., 1995).



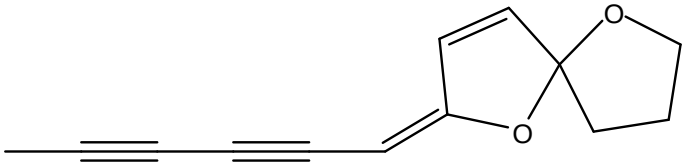
Phenylheptatriyne

Bidens pilosa
commons.wikimedia.org



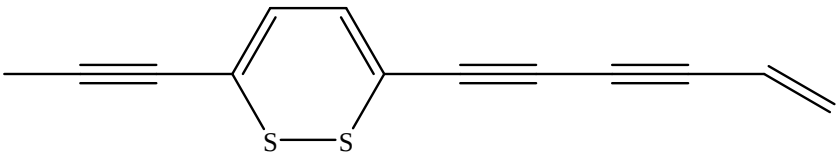
Capillin

Artemisia capillaris
commons.wikimedia.org



En-yn-dicycloether

Matricaria recutita
commons.wikimedia.org



Thiarubrine A

Ambrosia artemisiifolia
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Fig. 1.5: Examples of aromatic and cyclic polyacetylenes in Asteraceae.

1.2.4 Polyacetylenes from *Tagetes patula*

T. patula (French marigold) is an important aromatic herb (Fig. 1.6). It is a bushy annual, native to Mexico and other warmer parts of America and naturalized elsewhere in the tropics and subtropics. *T. patula* is about 15-45 cm high and characterized by large flower heads and alternate leaves (Priyanka et al., 2013). The plant is grown as an ornamental crop for its flowers which can be yellow, orange, and red depending on the variety (Bhattacharyya et al., 2010). Besides its use as an ornamental plant, the whole plant is reported to be used tradition-

ally for the treatment of cough and dysentery. The plant, in particular the flowers have hepatoprotective (Vasilenko et al., 1990), anti-inflammatory (Kasahara et al., 2002), nematicidal (Vasudevan et al., 1997), and insecticidal activities (Vasudevan et al., 1997). The oil from *T. patula* is used for the preparation of high grade perfumes. Whereas its carotenoid pigments are used in food coloring (Vasudevan et al., 1997). The majority of biological activities is related to the presence of thiophenes, the most studied compounds, which have been reported to be present in the whole plant (Mares et al., 1990; Hudson et al., 1993).

The biological activitities of thiophenes have increased the interest in these compounds. Thiophenes have antiviral, antibacterial, antifungal, nematocidal, and insecticidal properties which are enhanced by irradiation with long wavelength ultraviolet light (UVA, 320-400 nm) (Hudson et al., 1993). In fact, *Tagetes* plants are regarded as a source of natural pesticides (Marotti et al., 2010). Other chemical constituents found in different parts of *T. patula* also have biological activities, for example flavonoids and their glycosides (Vasudevan et al., 1997; Faizi et al., 2008), terpenoids, and carotenoids (Vasudevan et al., 1997; Garg et al., 1999).

Fig. 1.6: Cultivated *T. patula.*

Thiophenes are acetylenic compounds with one to three thiophene rings that are connected in α-position and carry an alkyne chain of one to six carbon atoms at the ortho-position (Bohlmann et al., 1973). Besides *T. patula*, these compounds occur also in other Tagetes species such as *T. minuta*, *T. erecta*, *T. laxa* and *T. mendocina* (Benavides and Caso, 1993; Talou et al., 1994; Marotti et al., 2010). Fig. 1.7 shows some thiophenes from the genus Tagetes. In *T. patula*, the major thiophenes are butenynyl-bithiophene (BBT) and acetoxybutynyl-

bithiophene (BBTOAc) which accumulate mainly in the roots whereas the concentration in leaves is low (Jacobs et al., 1994). Hydroxybutynyl-bithiophene (BBTOH) and α-terthienyl (α-T) are present in low amounts in all plant organs (Jacobs et al., 1994). PBT is the main component in the arial parts of the plant (Margl et al., 2002). Methyl-BBT and derivatives (Fig. 1.8) are present in negligible amounts compared to the other thiophenes in the plant roots (Croes et al., 1994). The level of thiophenes in *T. patula* increases as the plants grow older, reaching a maximum during the reproductive stages (Downum and Towers, 1983).

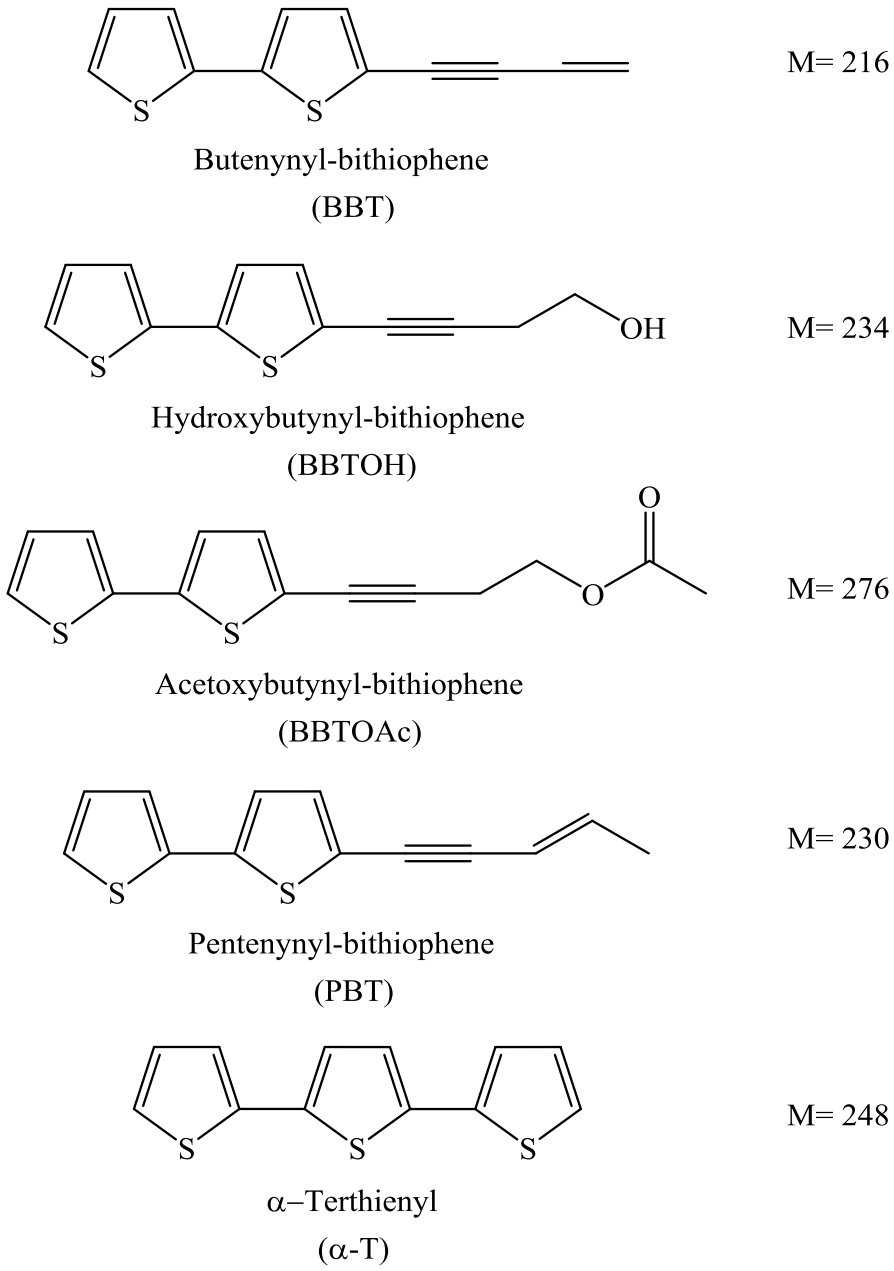


Fig. 1.7: Thiophenes in *T. patula* based on Margl et al. (2002) and Szarka et al. (2006). MW, molecular weight.

Due to the structural relationship between polyacetylenes and the naturally occurring thiophenes, it has been assumed that thiophenes in *T. patula* are synthesized from an oleic acid-derived polyacetylenic precursor, trideca-1-en-3,5,7,9,11-pentayne (T1e-3,5,7,9,11y). Labelling experiments with tritium with related plant species like *Echinops sphaerocephalus* L. and *Buphthalmum salicifolium* L. proved this assumption (Bohlmann and Hinz, 1965; Bohlmann et al., 1966). As indicated in Fig. 1.8, oleic acid is presumably converted to T1e-3,5,7,9,11y and T11e-1,3,5,7,9y by repeated steps of desaturation and chain shortening. The polyacetylenes are converted to thiophenes by addition of reduced sulfur to adjacent acetylenic groups of T1e-3,5,7,9,11y (Croes et al., 1994) (Fig. 1.8). The sulfur is donated from the sulfhydryl group of cysteine (Croes et al., 1994). The product, butenynyl-pentadiynyl-thiophene is the only monothiophene found in *T. patula* and considered the precursor of all bithienyls (Arroo et al., 1995).

Further biosynthetic step have been studied in *T. patula* hairy root cultures using labelling experiments (Arroo et al., 1995) (Fig. 1.8). After [^{35}S]butenynyl-pentadiynyl-thiophene was fed to the hairy roots, the radioactive label was found in all bithienyl thiophenes but not in α-T. This gives indication that butenynyl-pentadiynyl-thiophene is a precursor for the bithienyls but not for the terthienyls. When [^{35}S]methyl-BBT was fed to the roots, labelled O-acetylhydroxymethyl-BBT was formed, while neither labelled BBT nor other labelled bithienyls were detected. This showed that BBT is not formed by oxidative decarboxylation of methyl-BBT as previously proposed by Christensen and Lam. (1991b) but that methyl-BBT is an intermediate in the formation of O-acetylhydroxymethyl-BBT (Arroo et al., 1995). Hydroxymethyl-BBT is an intermediate formed by oxidation of the methyl group of methyl-BBT. Methyl-BBT and hydroxymethyl-BBT were detected in *T. patula* but only in very small amounts which indicates that the oxidation step occurs quickly (Arroo et al., 1995). When [^{35}S]O-acetylhydroxymethyl-BBT was fed, it was taken up by the roots but not converted (Arroo et al., 1995). This supports the proposed pathway in which O-acetylhydroxymethyl-BBT is produced from methyl-BBT through hydroxymethyl-BBT and indicates that O-acetylhydroxymethyl-BBT is a metabolically inactive end product (Arroo et al., 1995).

Feeding experiments with radiolabelled BBT in *T. patula* hairy roots (Arroo et al., 1995) led to the formation of [^{35}S]BBTOH, [^{35}S]BBTOAc, [^{35}S]dihydroxy-BBT and [^{35}S]di-(O-acetylhydroxy)-BBT. When [^{35}S]BBTOH was fed to the roots, the label was incorporated into BBTOAc at a high level. [^{35}S]BBTOAc was not converted upon feeding to the cultures (Arroo et al., 1995). This indicates that BBTOAc is produced from BBT via BBTOH. BBTOAc is considered a metabolically inactive end product (Arroo et al., 1995). This is in

agreement with Metschulat and Sütfeld (1987), who showed the activity of a specific acetyltransferase catalyzing the acetylation of BBTOH. Biosynthesis of PBT has not been investigated in previous labeling experiments (Arroo et al., 1995). We assume that T1,11e-3,5,7,9y could be a precursor for T1e-3,5,7,9,11y and T11e-1,3,5,7,9y. PBT could be produced from T11e-1,3,5,7,9y by addition of sulfur. Taken together, a biosynthetic scheme for thiophenes has been established based on labelling experiments, but the PBT biosynthesis pathway has not been investigated. The enzymes catalyzing the proposed reactions are largely unknown.

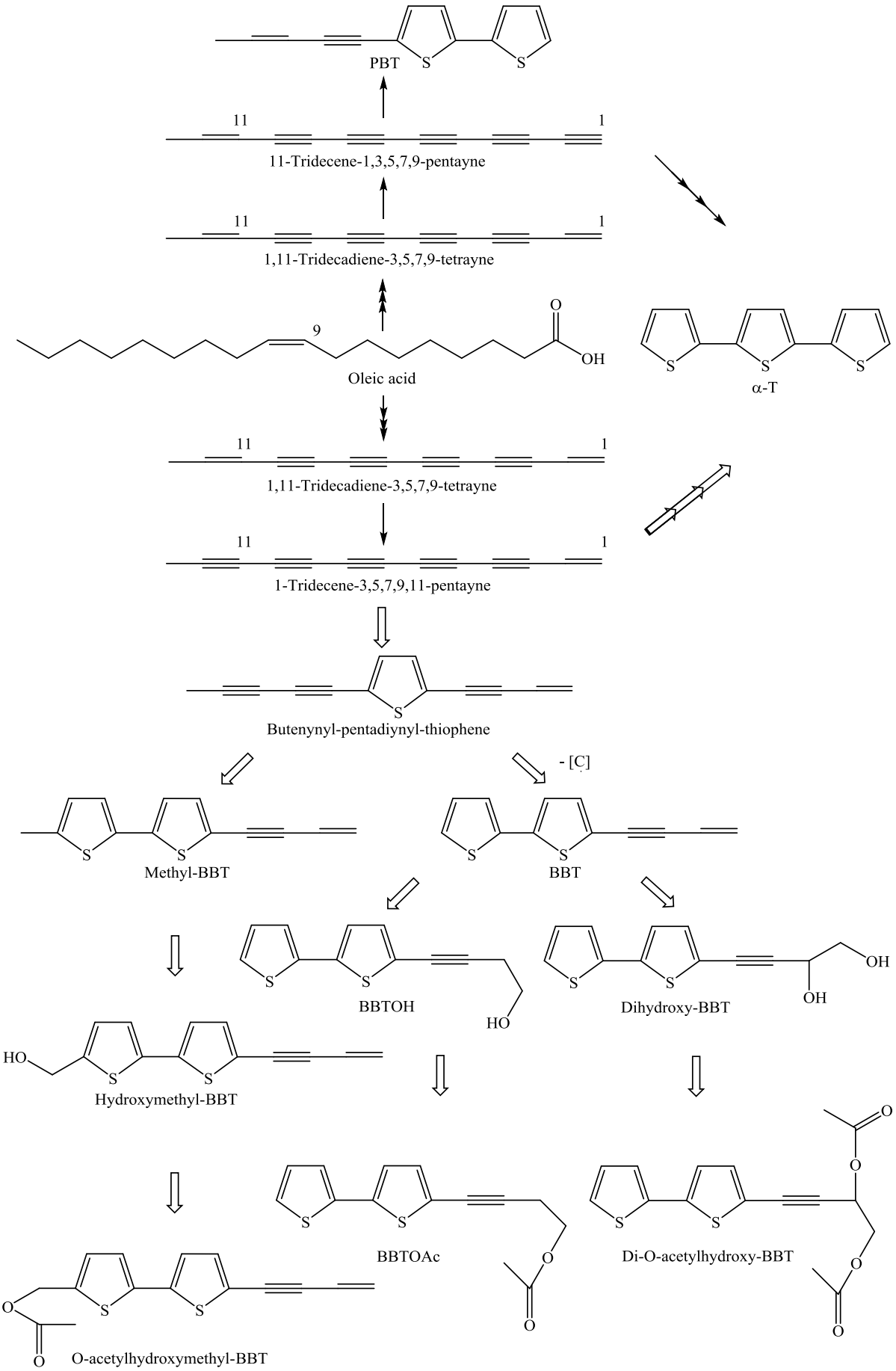

PBT
11
1
11-Tridecene-1,3,5,7,9-pentayne
11
1
1,11-Tridecadiene-3,5,7,9-tetrayne
9
O
OH
Oleic acid
α-T
11
1
1,11-Tridecadiene-3,5,7,9-tetrayne
11
1
1-Tridecene-3,5,7,9,11-pentayne
Butenynyl-pentadiynyl-thiophene
- [C]
Methyl-BBT
BBT
BBTOH
HO
Dihydroxy-BBT
OH
OH
HO
Hydroxymethyl-BBT
BBTOAc
O
Di-O-acetylhydroxy-BBT
O
O
O
O
O
O-acetylhydroxymethyl-BBT

Fig. 1.8: Scheme for the biosynthesis of thiophenes in *T. patula*. Reactions marked with block arrows were proposed by Arroo et al. (1995b) based on labelling experiments with [^{35}S]BPT, [^{35}S]methyl-BBT, [^{35}S]O-acetylhydroxymethyl-BBT, [^{35}S]BBTOH, [^{35}S]BBTOAc, [^{35}S]dihydroxy-BBT and [^{35}S]di-(O-acetylhydroxy)-BBT. The other arrows indicate hypothetic reactions.

1.2.5 Polyacetylenes from *Arctium lappa*

A. lappa (great burdock) is a perennial herb of the Asteraceae family (Fig. 1.9). The plant is about 3 m high in its second year, and distributed throughout the world. It is tolerant to salt, dryness, disease, and arid land so it can be grown under poor environmental conditions. The plant has been used in Europe, North America and Asia for centuries as therapeutic agent. It has been shown that the roots have hepatoprotective (Lin et al., 2002), anti-inflammatory (Lin et al., 1996), and antioxidative activity (Lin et al., 1996; Duh, 1998) due to the presence of caffeoylquinic acid derivatives, as well as antibacterial and antitumor activity (Lou et al., 2010; Predes et al., 2011). *A. lappa* is used in China as a traditional medicinal herb for treating colds, swelling of the throat and measles. *A. lappa* has been used also in dentistry due to its antimicrobial effect against oral microorganism (Gentil et al., 2006). The most utilized part is the dried first year roots, also fruits and leaves are used in which the plant stores most of its nutrients during the first year (Chan et al., 2011). *A. lappa* roots are used as food in China and Japan (Chan et al., 2011). The roots contain a mixture of aliphatic C_{13}-polyacetylenes and thiophenes (Washino et al., 1986; Takasugi et al., 1987) (Fig. 1.10).

Fig. 1.9: Cultivated *A. lappa*.

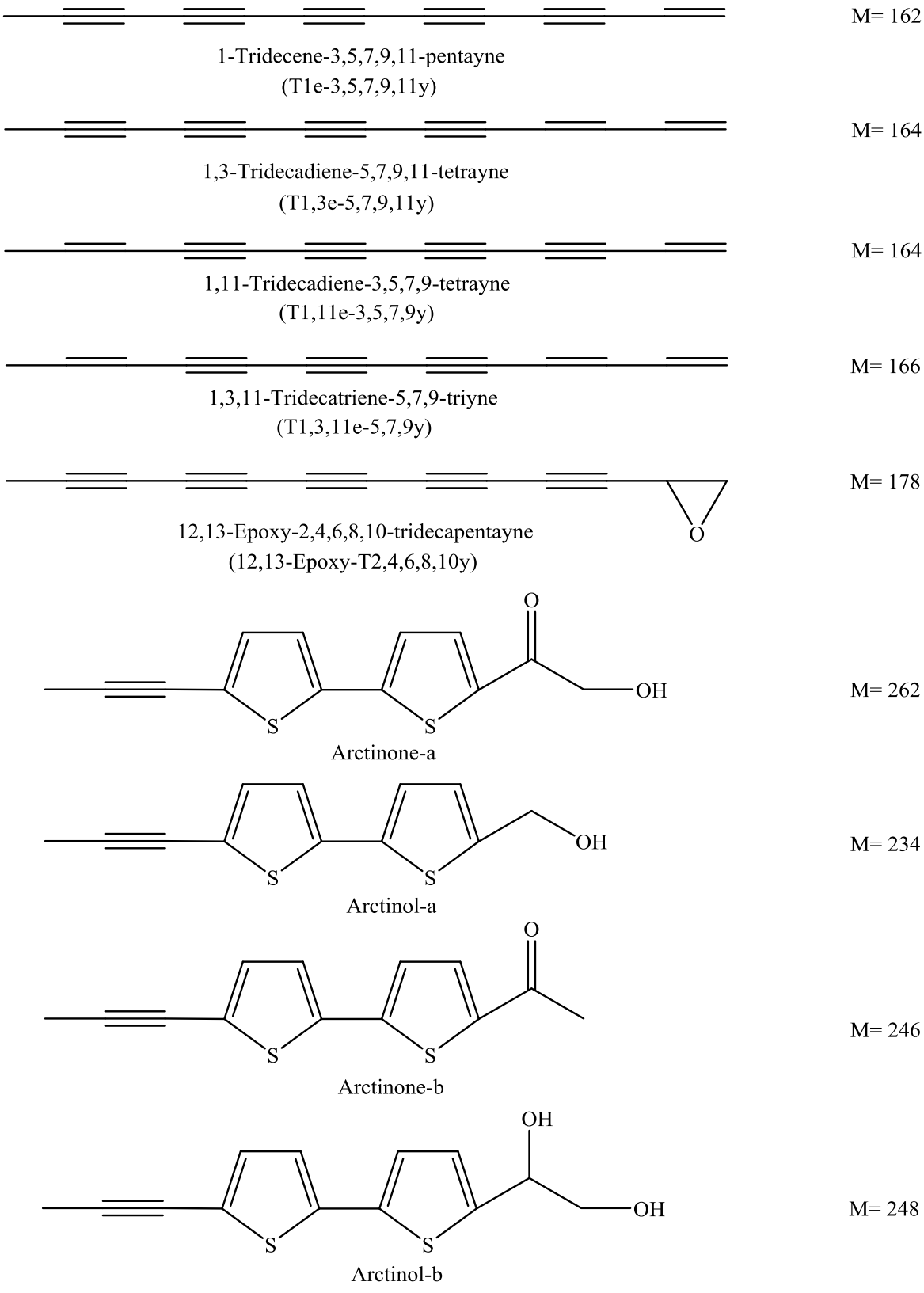


Fig. 1.10: Polyacetylenes in *A. lappa* based on Takasugi et al. (1987) and Binder et al. (1990a). MW, Molecular weight.

The biosynthesis pathway of polyacetylenes in *A. lappa* has not been studied previously. Based on the composition of polyacetylenes in *A. lappa*, a hypothetical pathway involving

hydrogenation and dehydration steps as well as an oxidation can be proposed (Fig. 1.11). T1e-3,5,7,9,11y has been identified as precursor of bithiophenes in *T. patula* (Arroo et al., 1995). Thus, it seems likely that thiophenes are formed from this compound also in *A. lappa.*

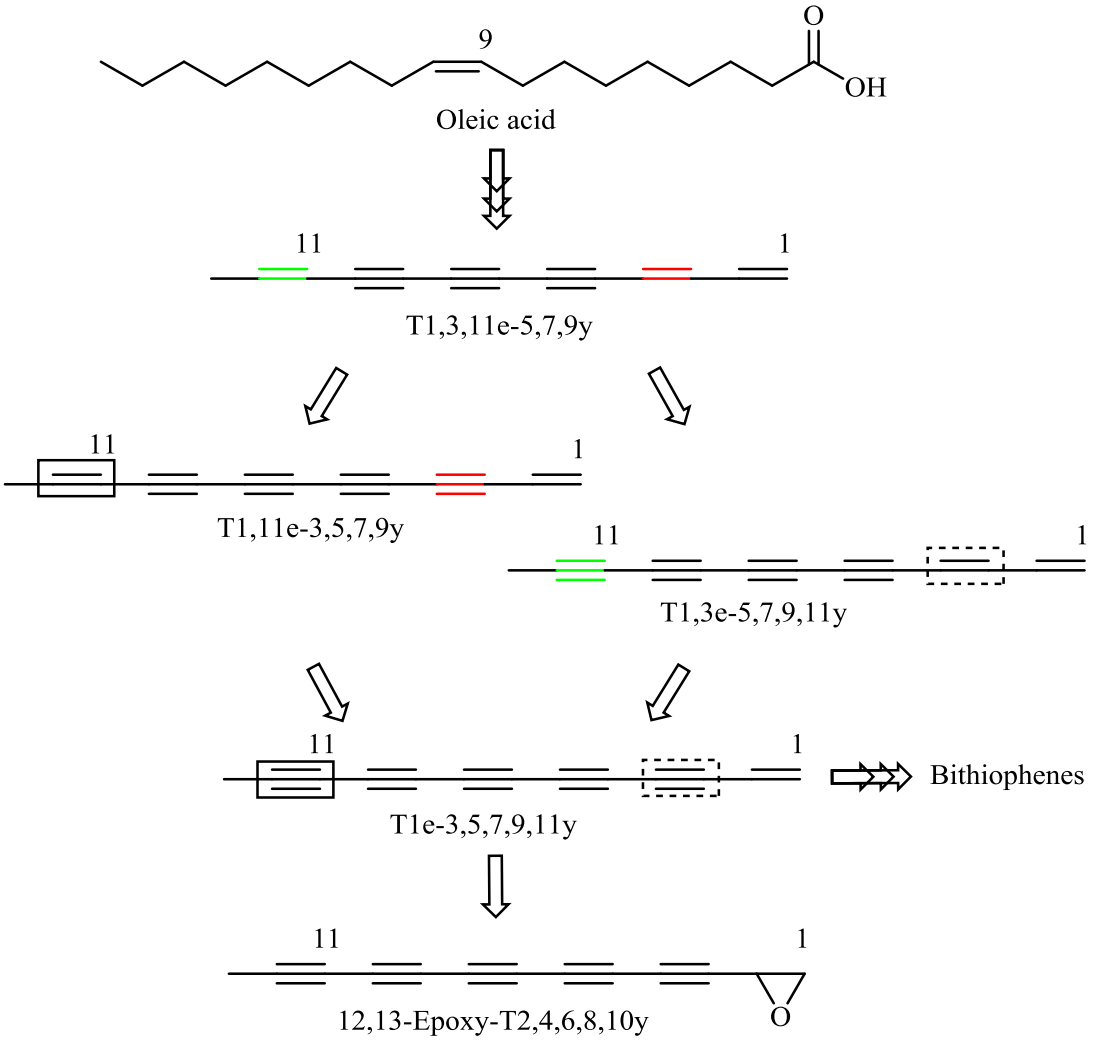


Fig. 1.11: Hypothetical pathway of the biosynthesis of polyacetylenes in *A. lappa.* The colored bonds (Red, Green) represent the chemical conversion of T1,3,11e-5,7,9y to T1,11e-3,5,7,9y, T1,3e-5,7,9,11y, respectively. The solid and dashed square represent the similarity between the product structure T1e-3,5,7,9,11y and the hypothetic intermediate T1,11e-3,5,7,9y and T1,3e-5,7,9,11y. T1e-3,5,7,9,11y is presumed to be a precursor for bithiophenes biosynthesis based on labeling experiments with related plants species (Bohlmann and Hinz, 1965; Bohlmann et al., 1966). The product structure 12,13-Epoxy-T2,4,6,8,10y could be formed by epoxidation of T1e-3,5,7,9,11y.

1.2.6 Polyacetylenes from *Carthamus tinctorius*

C. tinctorius commonly known as safflower is a highly branched, annual herbaceous plant of the Asteraceae family (Fig. 1.12). The plant is also known as false saffron, thistle saffron and dyer's saffron because it is used as substitute for the costly saffron. The plant is about 30

to 150 cm tall with spherical flower heads of usually yellow, orange or red color and has many sharp spines on the leaves and bracts (Dajue and Mündel, 1996). The plant grows around the world. It has a strong, deep taproot which enables it to take moisture and nutrients from considerable depth and grows in semi-arid, arid and salinity regions of the world (Dajue and Mündel, 1996). The plant is an important industrial crop, cultivated mainly for its seed which is used as birdseed and to produce edible oil. The oil has the highest levels of polyunsaturated fatty acids among all available oils (Kizil et al., 2008). In addition, the seeds contain plenty of polyphenolic compounds such as flavonoids and lignans as well as serotonins (Kim et al., 2007). The flowers contain pigments such as the water-soluble yellow dye, carthamidin, used mainly in colored juice and jelly and a water-insoluble red dye, carthamin, used in colored chocolate (Dajue and Mündel, 1996). Moreover, the dyes are used for cosmetics and fabric coloring. The flowers also contain flavonoids such as quercetin, kaempferol, and their glycosides (Kazuma et al., 2000). In traditional medicine, the plant has been used to relieve pains and problems associated with menstruation (Dajue and Mündel, 1996). Besides, it has been used in the treatment of several diseases such as cardiovascular disease, rheumatism, and chronic nephritis (Dajue and Mündel, 1996).

Fig. 1.12: Cultivated *C. tinctorius*.

Polyacetylenes of *C. tinctorius* are linear C_{13}-compounds (Fig. 1.13). The two polyacetylene diols safynol and dehydrosafynol have been referred to as phytoalexines as they have been isolated from plants infected with *Phytophthora drechsleri* or *P. megasperma* (Allen and Thomas, 1971b, 1972). It has been shown that safynol and dehydrosafynol have antifungal activity (Nakada et al., 1977) .

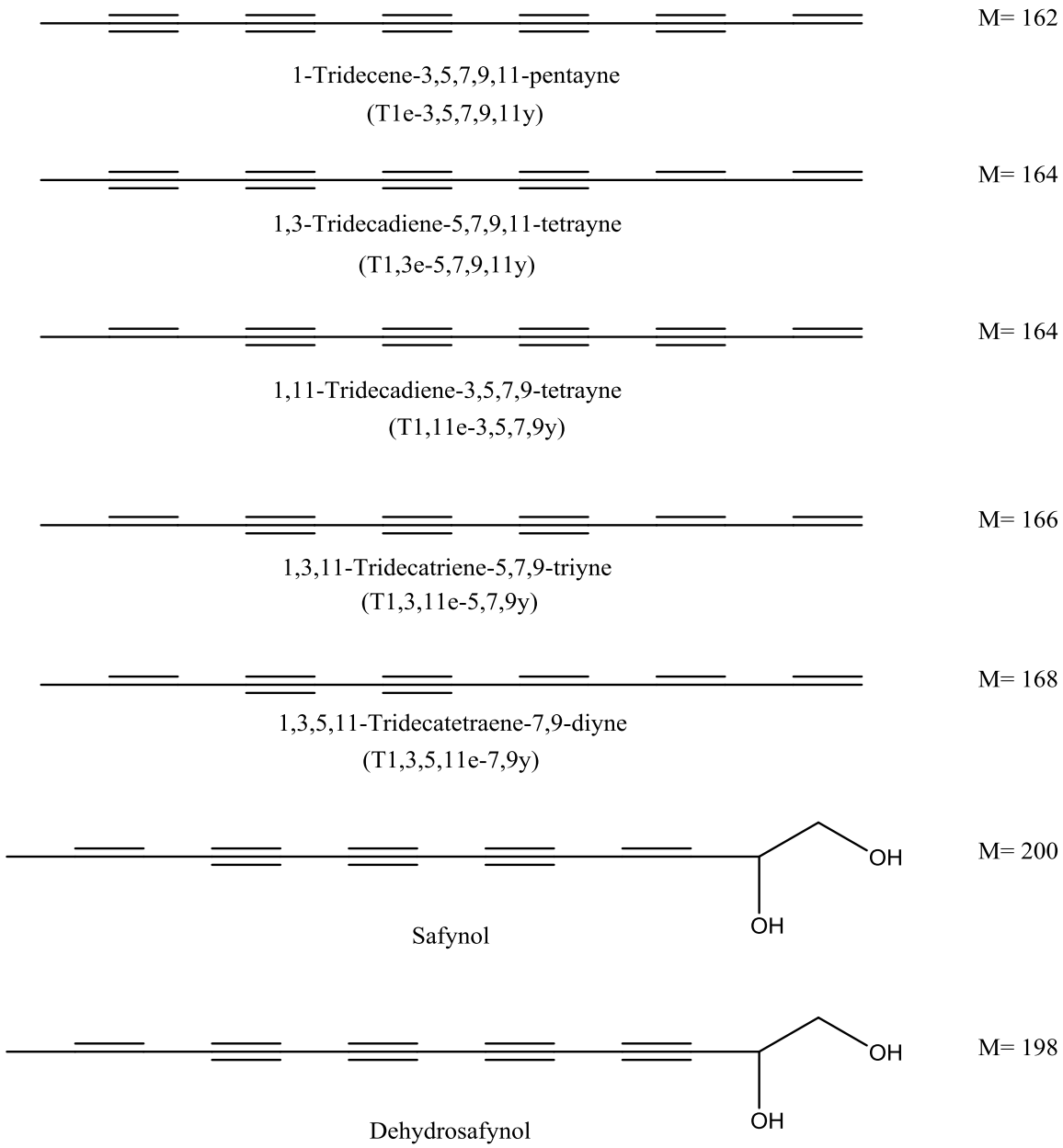


Fig. 1.13: Polyacetylenes in *C. tinctorius* based on Binder et al. (1990a). MW, Molecular weight.

The biosynthesis pathway of polyacetylenes in *C. tinctorius* has not been studied previously. Based on the composition of polyacetylenes in *C. tinctorius*, a hypothetical pathway can be proposed (Fig. 1.14).

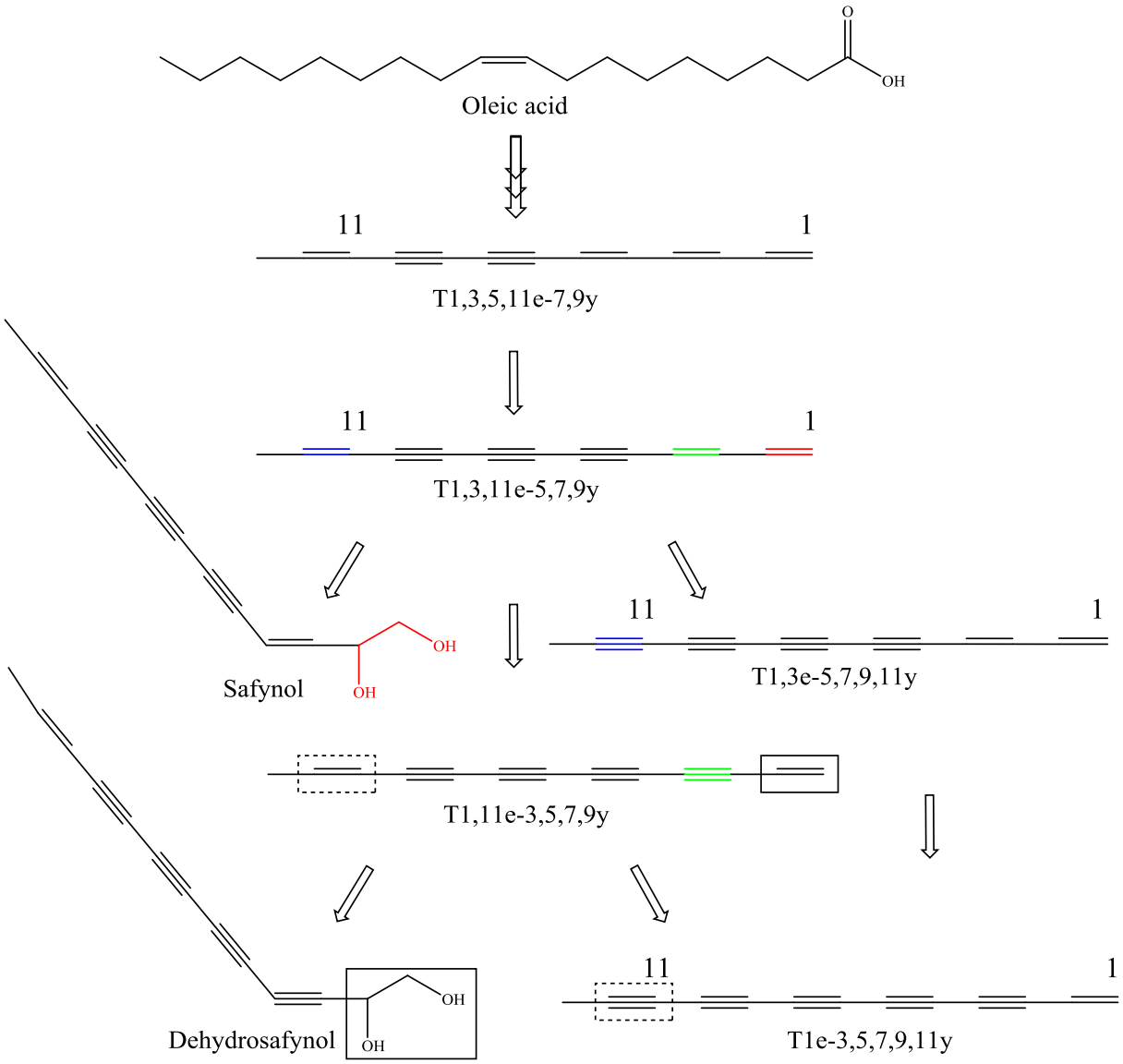


Fig. 1.14: Hypothetical pathway of the biosynthesis of polyacetylenes in *C. tinctorius*. The colored bonds (Red, Green, Blue) represent the chemical conversion of T1,3,11e-5,7,9y to safynol, T1,11e-3,5,7,9y and T1,3e-5,7,9,11y, respectively. The solid and dashed square represent the similarity between the hypothetic intermediate T1,11e-3,5,7,9y and the product structure dehydrosafynol and T1e-3,5,7,9,11y. T1e-3,5,7,9,11y could also be formed from T1,3e-5,7,9,11y.

1.3 Glucosinolates

1.3.1 The glucosinolate-myrosinase system

Glucosinolates are a group of low molecular weight, sulfur- and nitrogen-containing plant specialized metabolites found within the order *Brassicales*, which includes agriculturally important crops of the Brassicaceae family such as oilseed rape, vegetables like cabbage, broccoli, cauliflower, brussels sprouts, and the model plant *Arabidopsis thaliana* L. (Fahey et al., 2001). The glucosinolate molecule consists of a β-thioglucose moiety, a sulfated oxime and a variable side chain derived from amino acids (Halkier and Gershenzon, 2006) (Fig. 1.15).

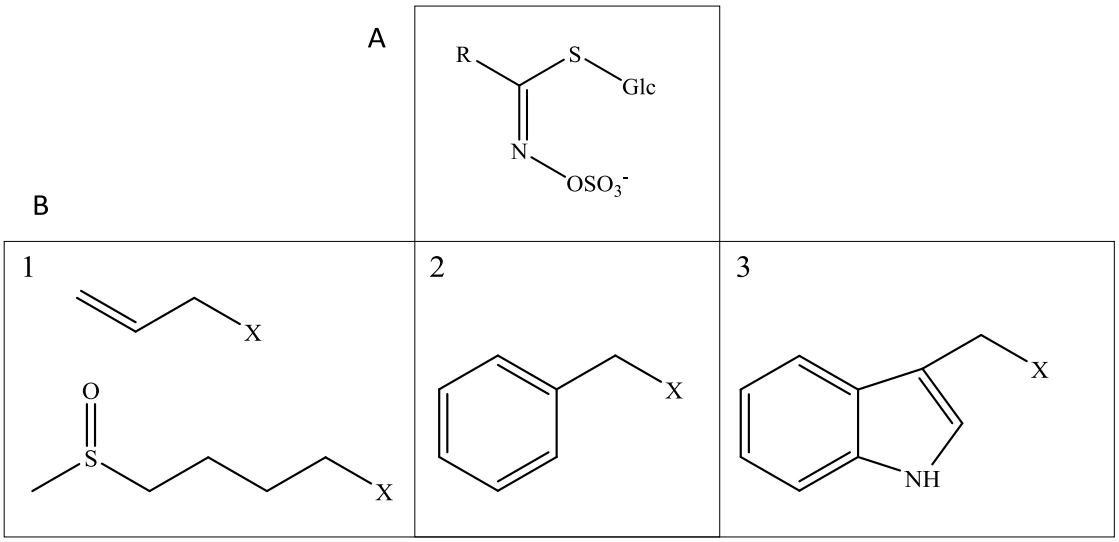


Fig. 1.15: Chemical structure of glucosinolates. A: The glucosinolate core structure (X) consists of a β-thioglucose moiety, a sulfated oxime and a variable side chain derived from amino acids; B: The variable side chain structures; (1) aliphatic, e.g. allyglucosinolate (above) and 4-methylsulfinylbutylglucosinolat (below); (2) aromatic, e.g. benzylglucosinolate; (3) indolic, e.g. indol-3ylmethylglucosinolate. Redrawn from: Winde and Wittstock (2011) with modifications.

Glucosinolate-containing plants also possess β-thioglucoside glucohydrolases known as myrosinases (EC 3.2.1.147) which are glycoproteins. Glucosinolates and myrosinases are spatially separated in intact plants. Myrosinases are found in idioblastic myrosin cells spread in most organs (Höglund et al., 1991) while glucosinolates are stored in vacuoles together with ascorbic acid (Grob and Matile, 1979). Upon tissue disruption as a result of wounding, insect, or pathogen attack, myrosinases come into contact with their substrates, the glucosinolates, leading to rapid generation of unstable thiohydroximate-O-sulfate intermediates (aglycones) that subsequently rearrange into isothiocyanates (Halkier and Gershenzon, 2006) (Fig. 1.16). Isothiocyanates, also known as mustard oils, are the predominant hydrolysis products. Isothiocyanates are the active substances of plant defense, because they are toxic to bac-

teria, fungi, insects and nematodes (Wittstock et al., 2003). Depending on the presence of additional proteins, the specifier proteins, and the glucosinolate side chain structure, alternative hydrolysis products such as simple nitriles, epithionitriles, and organic thiocyanates can also be formed (Wittstock and Burow, 2007). The hydrolysis products are responsible for the distinct taste and flavors of cruciferous plants while the parent glucosinolates lack a special odor or taste (Halkier and Gershenzon, 2006). The glucosinolate-myrosinase system plays an important role in plant defense against herbivores and pathogenes.

Human consumption of cruciferous vegetables is associated with beneficial effects including prevention of cardiovascular diseases and reduction of the risk to develop cancer (Traka and Mithen, 2009). A possible contribution of glucosinolates to these effects has been studied extensively. Among the degradation products with anticancer activity, sulforaphane, the isothiocyanate derived from 4-methylsulfinylbutylglucosinolate, induces apoptosis and stimulates phase II detoxification enzymes (Fahey et al., 2002). It also has antimicrobial effects against *Helicobacter pylori,* which causes stomach ulcers and is an important factor for development of gastric cancer (Fahey et al., 2002). 7-Methylsulfinylheptyl- and 8-methylsulfinyloctylisothiocyanates, derived from 7-methylsulfinylheptyl- and 8-methylsulfinyloctylglucosinolates respectively, are potent inducers of phase II enzymes which allow excretion of potential carcinogens (Traka and Mithen, 2009). Phenylethylisothiocyanate, derived from phenylethylglucosinolate, is a potent inhibitor of phase I enzymes which may activate carcinogens (Traka and Mithen, 2009). Benzylisothiocyanate, derived from benzylglucosinolate, possesses anti-inflammatory activity (Lee et al., 2009; Cheenpracha et al., 2010) and also has anticancer potential (Von Weymarn et al., 2006). Allyisothiocyanate, derived from allyglucosinolate, has anticancer effects (Zhang, 2010) and fungicidal activity (Zhang, 2010). A preparation of glucosinolate-containing plant material, leaves of *Tropaeolum majus* and roots of *Armoracia rusticana,* is available as an approved drug in Germany for treating of infection.

Although the glucosinolates and their hydrolysis products have beneficial effects to humans, these compounds reduce the quality of oil seed rape seed meal used as animal feed. Consumption of large amounts of rape seed meal has antinutritional and goitrogenic effects in animal which are associated with 5-vinyloxazolidine-2-thione, the spontaneous cyclization product of 2-hydroxy-3-butenylisothiocyanate, derived from 2-hydroxy-3-butenylglucosinolate, which can reach 80 % of total glucosinolates in rape seed (Fenwick et al., 1983).

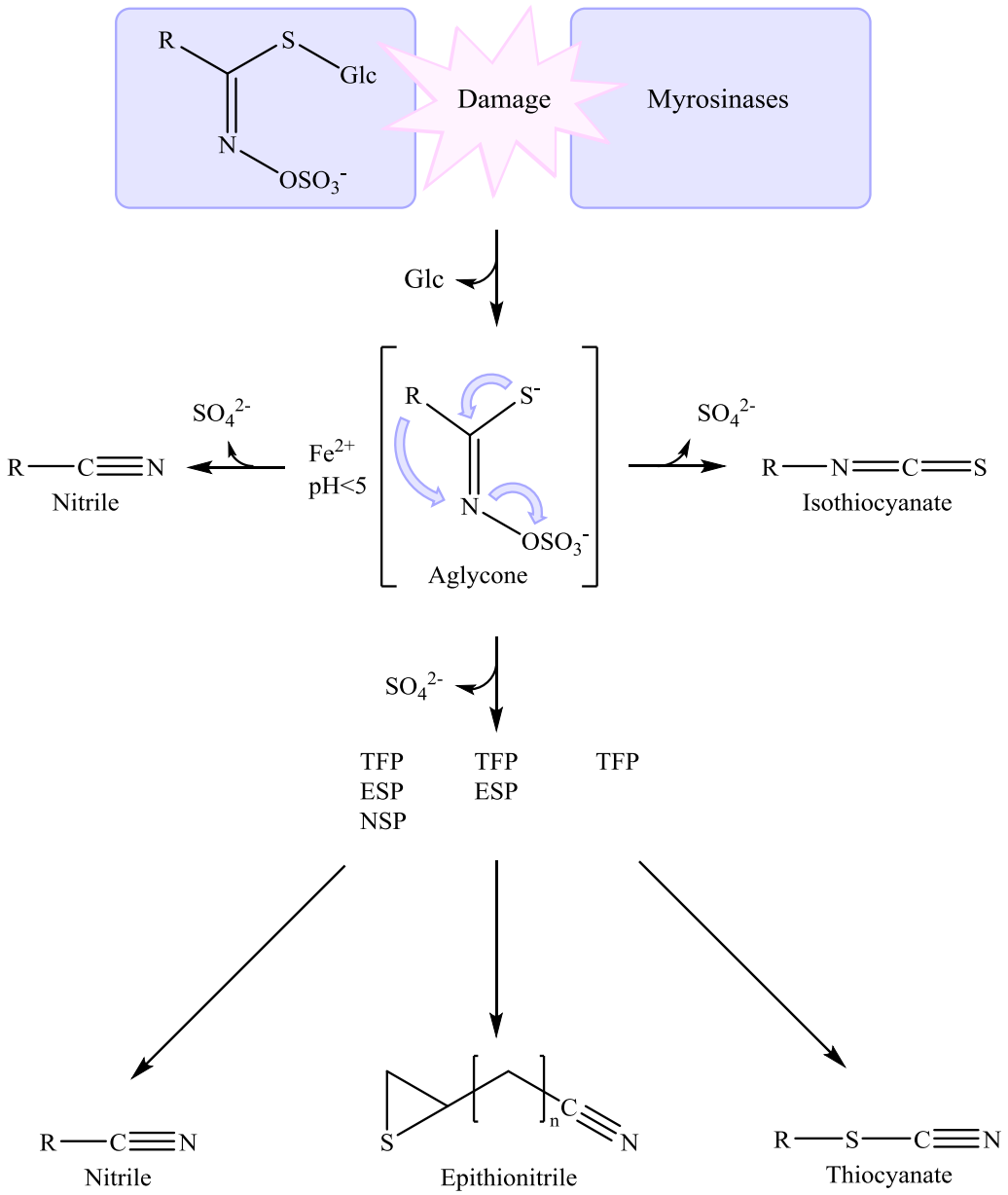


Fig. 1.16: The glucosinolate-myrosinase system. Upon tissue disruption, myrosinases come into contact with glucosinolates leading to rapid generation of unstable aglycones that subsequently rearrange into toxic isothiocyanates (right); simple nitriles may arise at pH<5 or when ferrous ions are present. In the presence of specifier proteins (TFP, ESP, NSP) other products are formed instead of isothiocyanates (Nitrile, Epithionitrile, Thiocyanate). TFP, thiocyanate-forming protein; ESP, epithiospecifier protein; NSP, nitrile-specifier proteins.

1.3.2 Glucosinolate biosynthesis

The glucosinolate core structure is derived from an amino acid precursor. Aliphatic glucosinolates are derived from alanine, methionine, leucine, isoleucine or valine, while indolic glucosinolates are derived from tryptophan. Other aromatic glucosinolates are derived from phenylalanine or tyrosine. The biosynthesis is divided into three stages: (I) chain extension of the starting amino acid, (II) formation of the glucosinolate core structure and (III) secondary

modification of the glucosinolate side chain (Sønderby et al., 2010). Most of our knowledge on enzymes involved in glucosinolates biosynthesis refers to enzymes identified in *A. thaliana* as outlined below.

For chain extension of the amino acid precursor (Fig. 1.17), the amino acid is first deaminated to the corresponding 2-oxo acid. This step occurs in the cytosol by a branched-chain amino acid aminotransferase (BCAT4) (Sønderby et al., 2010). The 2-oxo acid is transferred from the cytosol into the chloroplast by the plastidic BAT5 transporter (Gigolashvili et al., 2009). In the chloroplast, the 2-oxo acid is condensed with acetyl-CoA by a methylthioalkylmalate synthase (MAM) to form a 2-alkylmalate derivative. The 2-alkylmalate derivative is isomerized by isopropylmalate isomerase (IPMI) to form a 3-alkylmalate derivative that undergoes oxidative-decarboxylation by isopropylmalate dehydrogenase (IPMDH) to yield a 2-oxo acid with one more methylene group (Sønderby et al., 2010). The 2-oxo acid can be transaminated by BCAT3 to yield homomethionine which is transported to the cytosol or undergoes another cycle of chain elongation (Sønderby et al., 2010).

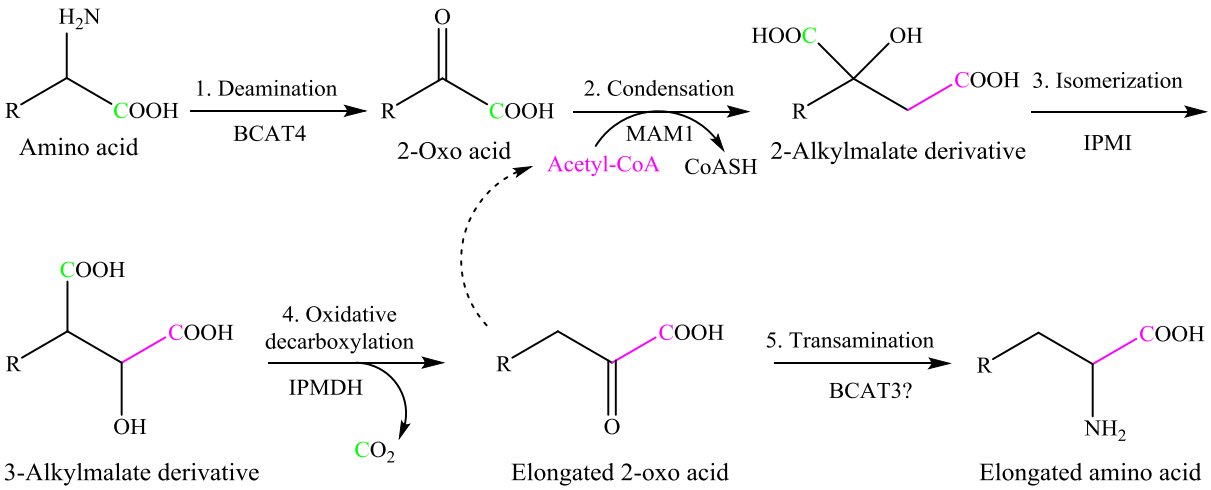


Fig. 1.17: Chain elongation of aliphatic amino acids. (1) Transamination to form 2-oxo acid; (2) Condensation with acetyl-CoA; (3) Isomerization; (4) Oxidative decarboxylation (5) Transamination. The 2-oxo acid can undergo up to 9 cycles of elongation (dashed curved arrow) before transamination for further glucosinolate synthesis. The carbon atom in the carboxyl group from acetyl-CoA is shown in purple while that from the precursor amino acid is shown in green. Abbreviation: R, aliphatic amino acid side chain; BCAT4, branched-chain amino acid aminotransferase; MAM1, methylthioalkylmalate synthase; IPMI, isopropylmalate isomerase; IPMDH, isopropylmalate dehydrogenase.

The synthesis of the core structure involves principle intermediates common to all glucosinolates (Fig. 1.18). The first two steps are catalyzed by cytochrome P450 enzymes belonging to the CYP79 and CYP83 families, respectively (Sønderby et al., 2010). The former convert

the amino acid to an aldoxime, and this step is a key step in glucosinolate biosynthesis. CYP79A2 uses phenylalanine as a substrate (Wittstock and Halkier, 2000), CYP79B2 and CYP79B3 use tryptophan as a substrate (Mikkelsen et al., 2000), and CYP79F1 and CYP79F2 use chain-elongated methionine derivatives as a substrates (Hansen et al., 2001). CYP79F1 converts short-chained methionine derivatives while CYP79F2 converts long-chained methionine derivatives (Hansen et al., 2001; Chen et al., 2003). Cytochromes P450 belonging to the CYP83 family oxidize aldoximes to activated aldoximes (nitrile oxides or aci-nitro compounds). CYP83B1 acts specifically on aromatic aldoximes while CYP83A1 acts on aliphatic aldoximes (Bak and Feyereisen, 2001). The activated aldoximes are conjugated with glutathione as sulfur donor either spontaneously or catalyzed by unspecific glutathione S-transferases (GSTs) (Mikkelsen et al., 2010). Next, γ-glutamyl peptidase 1 (GGP1) hydrolyses the amide with which the glutamyl residue is fixed. The resulting product, S-alkylthiohydroximates, has a free amino group and can thus be cleaved by a C-S lyase (Sur1) to give a thiohydroximate (Mikkelsen et al., 2004; Geu-Flores et al., 2009a). This is subsequently glucosylated by a soluble thiohydroximate glucosyltransferase of the UGT74 family to produce a desulfoglucosinolate (Sønderby et al., 2010). The final step of glucosinolate biosynthesis is the sulfation of the desulfoglucosinolate. This reaction is catalyzed by sulfotransferases (SOT) which transfer a sulfate group from 3'-phosphoadenosine 5'-phosphosulfate (PAPS) to a hydroxylated substrate. The desulfoglucosinolates derived from phenylalanine or tryptophan are substrate for SOT16, while desulfoglucosinolates derived from methionine are substrate for SOT17 and SOT18 (Sønderby et al., 2010).

After the glucosinolate core structure is synthesized, the glucosinolates can be subjected to modifications of the side chain which play an important role in determining the spectrum and biological activity of glucosinolate degradation products. Flavin-monooxygenase (FMO) enzymes, $FMO_{GS\text{-}OX1}$ to $FMO_{GS\text{-}OX5}$ have been identified to catalyze the S-oxygenation from methylthioalkyl- to methylsulfinylalkylglucosinolates (Hansen et al., 2007; Li et al., 2008). $FMO_{GS\text{-}OX1\text{-}4}$ have broad substrate specificity while $FMO_{GS\text{-}OX5}$ accepts only long-chain 8-methylthiooctylglucosinolate (Li et al., 2008). Methylsulfinylalkylglucosinolates can be converted to either the alkenyl- or the hydroxyalkyl- form by 2-oxoglutarate-dependent dioxygenases, AOP2 and AOP3, respectively (Kliebenstein et al., 2001). 2-Hydroxybut-3-enyl-glucosinolate, which is responsible for the bitter taste of Brassica vegetables, is produced from hydroxylation of but-3-enyl glucosinolate by the 2-oxoacid-dependent dioxygenase encoded by *At2g25450* (*GS-OH*) (Hansen et al., 2008). Cytochrome P450 monooxygenases of the CYP81 family hydroxylate indol-3-yl-methylglucosinolate to 4-hydroxyindol-3-ylmethyl-

glucosinolate and/ or 1-hydroxyindol-3-yl-methylglucosinolate. Upon methylation of the four hydroxy group by the O-methyltransferases IGMT1 and IGMT2, 4-methoxyindol-3-yl-methylglucosinolate is produced (Pfalz et al., 2011) which plays a role in defense responses to pathogenes (Bednarek et al., 2009; Clay et al., 2009). Taken together, glucosinolate biosynthesis is accomplished through at least seven steps (i.e. the steps required for core structure biosynthesis). For example, in biosynthesis of benzylglucosinolate, phenylalanine is the amino acid precursor without prior side chain modification and no modifications are required after core structure biosynthesis. However, most aliphatic glucosinolates are derived from chain-elongated amino acid or carry otherwise modified side chains, and therefore their biosynthesis requires more biosynthetic steps.

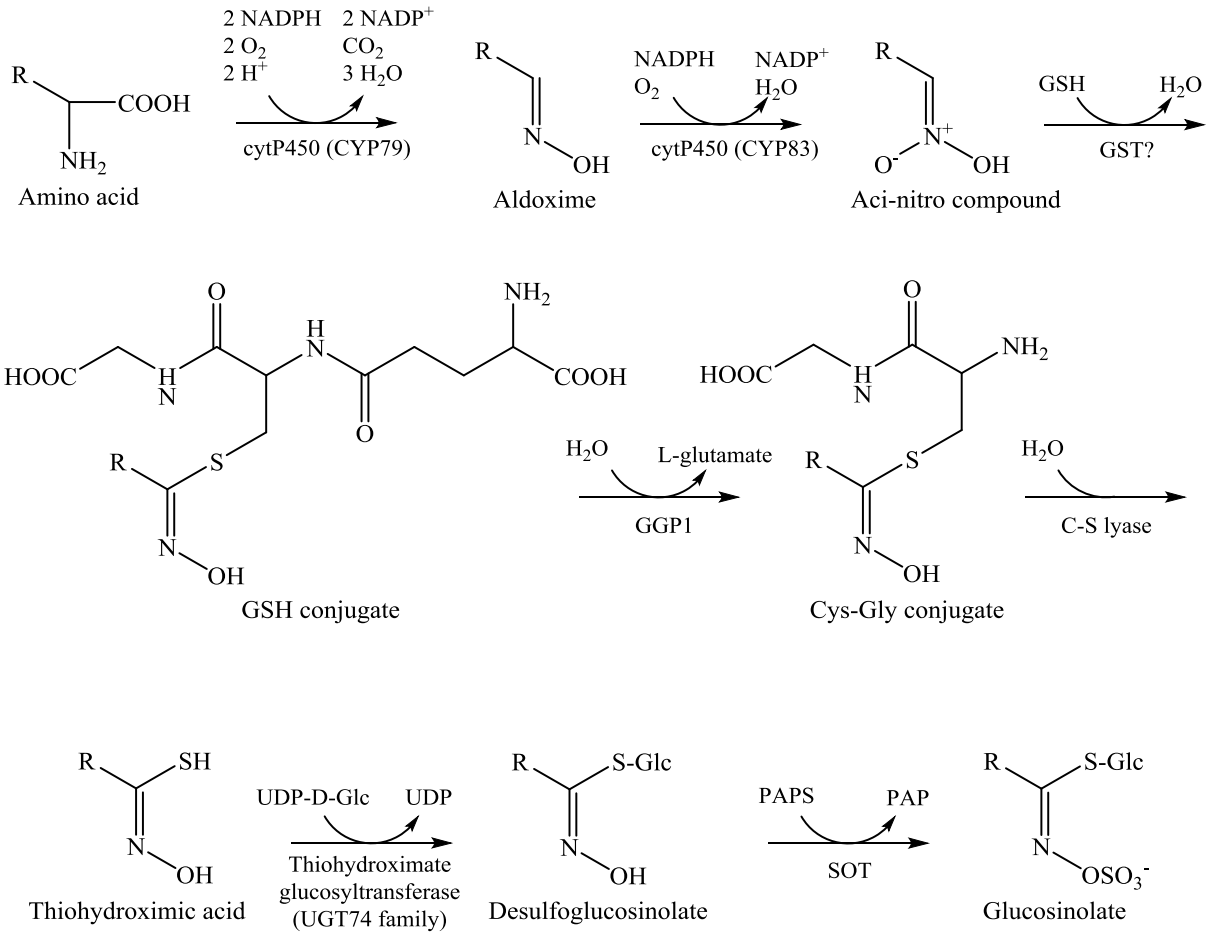


Fig. 1.18: Biosynthesis of glucosinolate core structure. The synthesis of glucosinolate core structures from the elongated or non-elongated amino acid is involved intermediates prevalence to all glucosinolates. Abbreviation: R, amino acid side chain; GST, glutathione S-transferase; GGP1, γ-glutamyl peptidase 1; SOT; sulfotransferase.

1.3.3 Genetic engineering of glucosinolate biosynthesis in heterologous hosts

The glucosinolate biosynthetic pathway has been transferred to heterologous hosts in the course of biosynthetic studies, but also in order to develop biotechnological production platforms or to explore glucosinolates in pest management strategies.

Biosynthesis of benzylglucosinolate in *N. benthamiana* (which does not normally produce glucosinolates) required transfer of six genes, namely CYP79A2, CYP83B1, GGP1, Sur1, UGT74B1, and AtSOT16 (see appendix 7.3) (Geu-Flores et al., 2009a; Møldrup et al., 2011). Upon transient expression under control of the Cauliflower mosaic virus 35S (CaMV35S) promoter, levels of about 0.57 nmol/mg FW (233 ng/mg FW) were obtained (Geu-Flores et al., 2009a). Benzylglucosinolate levels obtained in stably transformed lines of *N. tabacum* were in a similar range (0.5 nmol/mg FW) (Møldrup et al., 2012).

Indolylglucosinolate biosynthesis has been engineered in *N. benthamiana* (Pfalz et al., 2011) as well as in *S. cerevisiae* (Mikkelsen et al., 2012). Transfer of six genes through transient transformation of *N. benthamiana* resulted in levels of 0.2 nmol/mg FW (89 ng/mg FW) indol-3-yl-methylglucosinolate. Stable integration of the biosynthetic genes into the genome of *S. cerevisiae* resulted in production 1.07 mg/l indol-3-yl-methylglucosinolate. Indolylglucosinolate was secreted to the medium and only small amounts were detected intracellularly.

The aliphatic glucosinolate 4-methylsulfinylbutylglucosinolate (glucoraphanin), the major glucosinolate in broccoli derived from dihomomethionine was produced in *N. benthamiana* by transient expression of 13 genes (five genes responsible for chain elongation pathway, seven genes for the glucosinolate core pathway and one gene for side chain modification). 4-methylsulfinylbutylglucosinolate was accumulated to about 0.04 nmol/mg FW (17 ng/mg FW) (Mikkelsen et al., 2010). Recently, the methionine chain elongation pathway has been introduced to *E. coli* offering new possibilities for aliphatic glucosinolate production (Mirza et al., 2016).

1.3.4 The viral 2A polycistronic open reading frame as a tool to express multiple genes in eukaryotic cells

Picornaviruses encode all of their proteins in a single open reading frame (ORF). Due to fast intramolecular primary co-translational cleavages mediated by virus-encoded proteinases, the full-length translation product is not detectable in virus infected cells (Belsham, 2005). The picornaviral ORF can be divided into the P1, P2 and P3 regions which encode for capsid proteins (P1) and replication proteins (P2 and P3) (Luke, 2012). Some picornaviruses possess an additional L-region (L^{Pro}) at the 5` end of the ORF (Luke, 2012). Consecutive sections of the P1, P2, and P3 regions are designated 1A-1D (P1), 2A-2C (P2), and 3A-3D (P3)

(Fig. 1.19) (Luke, 2012). While some of the cleavages occur by autoproteolytic processing, the separation between P1 and P2 is due to ribosomal skipping mediated by the so-called 2A region in certain subgroups of the picornoviruses such as aphtho and cardioviruses (Donnelly et al., 2001).

Ribosomal skipping has first been studied in foot and mouth disease virus (FMDV), an aphthovirus. In the FMDV-ORF, the 2A region encodes a peptide of only 18 amino acids with the C-terminal sequence ESNPG (Donnelly et al., 2001). When this sequence was inserted between two reporter genes together with the first codon of the 2B region (coding for Pro), translation *in vitro* resulted in two separate polypeptides (Halpin et al., 2001). On translation of a polyprotein with the 2A region, the Pro-Gly peptide bond at the C-terminus of 2A is synthesized. The peptidyl-tRNA is translocated from the A to the P site of the ribosome. This allows binding of the following prolyl-tRNA (2B) to the A site. However, the C-terminal part of the 2A-peptide (-ESNPG-) is sterically hindered within the ribosomal peptidyl transferase center. This inhibits peptide bond formation between the nascent peptide with C-terminal glycine at the P site and the prolyl-tRNA at the A site. The prolyl-tRNA exits the A site. Then, the releasing factor eRF1 binds to the A site. The ester bond between the peptidyl residue and the tRNA is hydrolyzed and the upstream nascent protein with the 2A peptide at the C-terminus is released. The prolyl-tRNA may now re-enter the A site and be translocated by eEF2 into the P site. The translation of the downstream product can continue and it will be released with the prolyl residue at the N-terminus. Mutation of the N-terminal proline residue of 2B leads to impairment of the 2A skipping mechanism and results in the synthesis of uncleaved polyproteins (Donnelly et al., 2001).

The 2A system is active in all eukaryotic cells that have been tested while it is apparently inactive when used in prokaryotes (Donnelly et al., 1997). When the 2A sequence of FMDV is introduced between genes of a polycistronic construct for expression in eukaryotic cells, the primary translation product will be split behind the 2A region. Thus, the 2A system can be used to achieve expression of multiple, discrete proteins derived from a single ORF driven by a single promoter. As the repeated use of a strong promoter may lead to transgene silencing, the 2A system may help to avoid silencing upon overexpression of transgenes. The 2A system is widely used as a tool for co-expression of genes in biomedicine and biotechnology. As an example, the 2A system has been used to generate the “golden rice” by overexpression of β-carotene biosynthetic pathway genes in rice endosperm (Ha et al., 2010) and to achieve production of indol-3-yl-methyl glucosinolate and benzylglucosinolate in yeast and tobacco (Geu-Flores et al., 2009a; Pfalz et al., 2011; Mikkelsen et al., 2012).

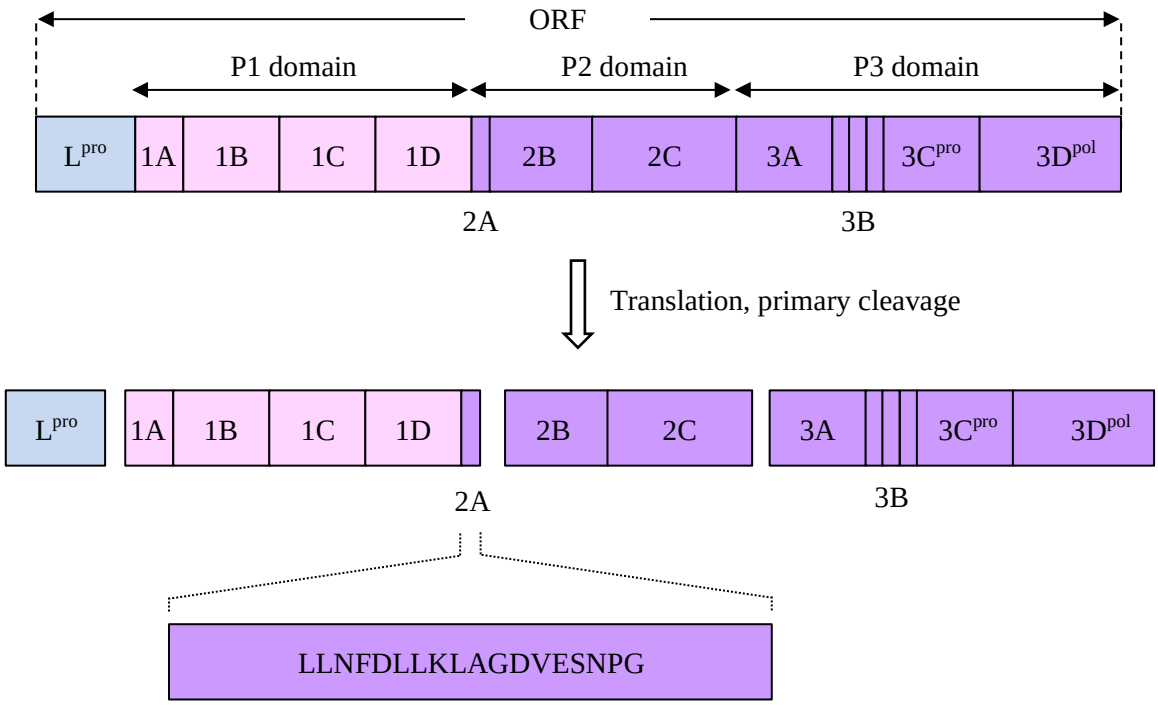


Fig. 1.19: Structure of FMDV-ORF and translational products after primary cleavage. The FMDV polyprotein consist of three domains: N-terminal capsid protein P1 (pink box) and the middle and C-terminal nonstructural proteins P2 and P3 (violet box), respectively. L^{pro} cleaves at its C-terminus, $3C^{pro}$ cleaves between (2BC) and 3A, and 2A mediates cleavage at its C-terminus by inducing ribosomal skipping. The 2A sequence of FMDV is given. Abbreviation: Pro, proteinase; Pol, polymerase. Redrawn from: Luke (2012) with modifications.

1.4 Aim of this work

In our search for a biological system for studies on polyacetylene biosynthesis in the Asteraceae, one goal of this study was to identify a tissue active in the biosynthesis of thiophenes or aliphatic polyacetylenes. Three polyacetylene-containing species, *T. patula*, *A. lappa*, and *C. tinctorius* were selected to be investigated with respect to the following objectives:

- to compare polyacetylene content and composition in above- and below-ground organs of soil-grown plants
- to establish root, hairy root, callus, and callus cell suspension cultures
- to determine growth and polyacetylene content and composition of these *in vitro* cultures
- to test if polyacetylene biosynthesis can be induced by elicitors such as MeJ, chitosan, vanadyl sulfate, and copper sulfate.

Despite their use in biosynthetic studies and as a host for production of therapeutic proteins, callus cell suspension cultures have not been explored as a host for heterologous expression of a whole biosynthetic pathway of specialized plant metabolies. Therefore, a second goal of this study was to test and optimize techniques for heterologous expression of multiple genes in callus cell suspension cultures of *Daucus carota.* The well characterized pathway of benzylglucosinolate biosynthesis was selected to be transferred to carrot cells. Carrot cell suspension cultures were investigated with the following objectives:

- to determine growth characteristics
- to optimize conditions for *A. tumefaciens*-mediated transformation of the cells with two different constructs
- to establish a method for clonal selection
- to test and optimize techniques for cryopreservation of carrot cell suspension cultures.

2 Experimental

2.1 Chemicals and biochemicals

Chemicals, reagents and solvents were purchased from the following companies unless otherwise mentioned: Sigma-Aldrich (St. Louis, USA), Carl Roth (Karlsruhe, Germany) and Acros (Geel, Belgium). The enzymes and molecular biology reagents were purchased from Thermo Fisher Scientific Inc. (Waltham, USA). Deionized water was supplied from Milli-Q water purification system (Sartorius, Germany), and used in preparing all aqueous solutions used in the study.

2.2 Material and methods for polyacetylene analysis

2.2.1 Seed source

- ***Tagetes patula* (var. Orange Boy):** Seeds were purchased from Samenhaus Knieke, Braunschweig, Germany.
- ***Arctium lappa***: Seeds were purchased from Stickysticky ethnobotanicals/eBay Shops, Great Britain. Other seeds were a gift from the Botanical Garden of Technische Universität Braunschweig, Germany, and others were donated from the Botanical Garden of Karl-Franzens-Universität Graz, Austria.
- ***Carthamus tinctorius* (var. Goldschopf)**: Seeds were purchased from Samenhaus Knieke, Braunschweig, Germany.

2.2.2 Soil-grown plants

Unless otherwise stated, the plants were grown from seeds in the greenhouse of the Institute of Pharmaceutical Biology, Technische Universität Braunschweig, Germany (Fig. 2.1) on flowers soil (Gartenkraft®) that autoclaved at 101°C for 20 min. The plants were watered regularly. The roots, flowers, leaves, flower buds, stems, primary roots, and secondary roots were harvested separately. Roots were washed under running tap water. The harvested parts of the plant were frozen with liquid nitrogen and dried in the lyophilizer or preserved at -20°C until use. The planting and harvesting times of the three Asteraceae species were as described below:

- ***T. patula* (var. Orange Boy):** The seeds were sown in early March 2011 and harvested at different times, when the seedlings were twelve-day-old, one-month-old and also during the full flowering period at the end of May 2011.
- ***A. lappa*:** The seeds were dispersed in January 2012 and plants grown in a growth cabinet at 22°C and 16 h light. At the end of February the plants were transferred to the

greenhouse and harvested in April 2012. Fifteen-day-old and one-month-old seedlings were also harvested.

- ***C. tinctorius* (var. Goldschopf):** The seeds were sown in February 2012 in a growth cabinet at 22°C and 16 h photoperiod. At the end of March the plants were transferred to the greenhouse and harvested in May 2012 during the full flowering period. Fifteen-day-old and one-month-old seedlings were also harvested.

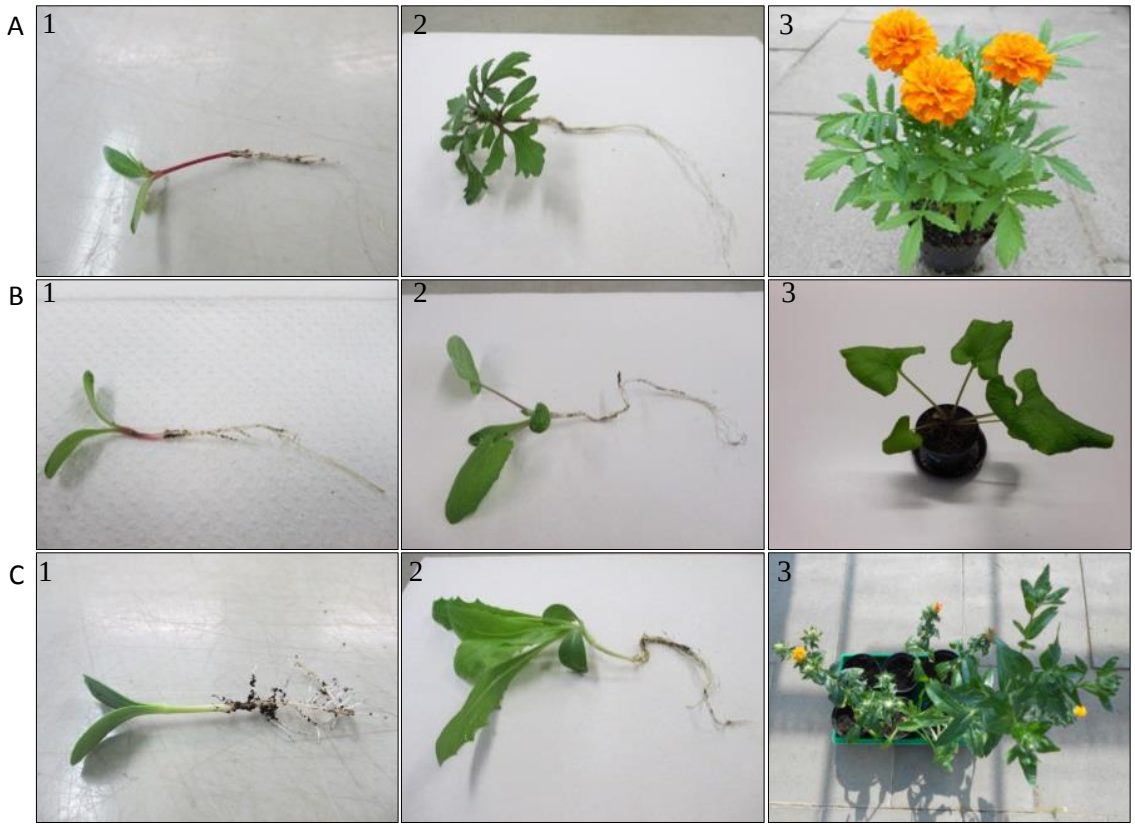


Fig. 2.1: Stages of experimental plants analyzed. A: *T. patula*; B: *A. lappa*; C: *C. tinctorius*; (1) Fifteen-day-old seedlings; (2) One-month-old plants; (3) About three-month-old plants.

2.2.3 Aseptically grown plants

2.2.3.1 Seed sterilization and treatment

The seeds were surface sterilized under the clean bench by immersion and shaking in 70 % ethanol for not more than 2 minutes followed by treatment with 3 % (v/v) sodium hypochlorite for 4, 8 or 12 min. They were finally rinsed twice with sterile water and then the seeds were aseptically germinated in Murashige and Skoog medium (solid MS medium, Tab. 2.1) (Fig. 2.2). The cultures were maintained at 20°C under a 16 h photoperiod. The *in vitro* germination was as follows:

- ***T. patula*:** Most of the seeds germinated after 7 days.

- ***A. lappa*:** The seeds from Great Britain germinated within three weeks. No fungal contamination was observed. The other seeds were contaminated with fungi although the sterilization of the seeds was intensified by increasing the seeds soaking period in 3 % (v/v) sodium hypochlorite to 20 and 30 min and also by treating the seeds with 1N sulfuric acid for 15 min or 16 h.
- ***C. tinctorius*:** The seed coat of *C. tinctorius* is thick. In order to overcome dormancy, the seeds were soaked for two hours in warm water at about 50 °C before sterilization. They germinated after about one week.

Fig. 2.2: Aseptically grown seedlings of the Asteraceae plants. A: *T. patula*; B: *A. lappa*; C: *C. tinctorius*.

Tab. 2.1: Composition of Murashige and Skoog (MS) medium.

Medium	Compositions	Per 1 l
MS medium	**1. Macro elements:**	
	NH_4NO_3	1650 mg
	KNO_3	1900 mg
	$MgSO_4 . 7 H_2O$	370 mg
	KH_2PO_4	170 mg
	$CaCl_2 . 2H_2O$	440 mg
	Fe EDTA	40 mg
	2. Micro elements:	
	$MnSO_4 . 4H_2O$	10 mg
	H_3BO_3	3 mg
	$ZnSO_4 .7H_2O$	2 mg
	$Na_2MoO_4 . 2H_2O$	2.5 mg
	$CuSO_4 . 5H_2O$	0.025 mg
	$CoCl_2 . 6H_2O$	0.025 mg
	KI	0.075 mg
	3. Vitamins:	
	Nicotinic acid	1 mg
	Pyridoxin HCl	1 mg

	Thiamin HCl	10 mg
	Myo-Inositol	100 mg
	4. Carbohydrates:	
	Sucrose	30 g
	➢ pH-adjusted to 5.8 with 0.5 N NaOH, autoclaved.	
Solid MS medium	MS medium with 0.8 % (w/v) agar	
	➢ Agar added prior to autoclaving	

2.2.3.2 Plant growth and maintenance

After germination, each seedling was transferred to a 100 ml flask containing 30 ml solid MS medium (Tab. 2.1). When the plants became bigger, they were moved to bigger flasks (250 ml) containing 50 ml of the same medium (Fig. 2.3). One-month-old plant was transferred to fresh medium. The plant was used as a source for establishment of cell culture. To maintain *C. tinctorius* for more than two weeks, it had to be transferred to ½ MS medium.

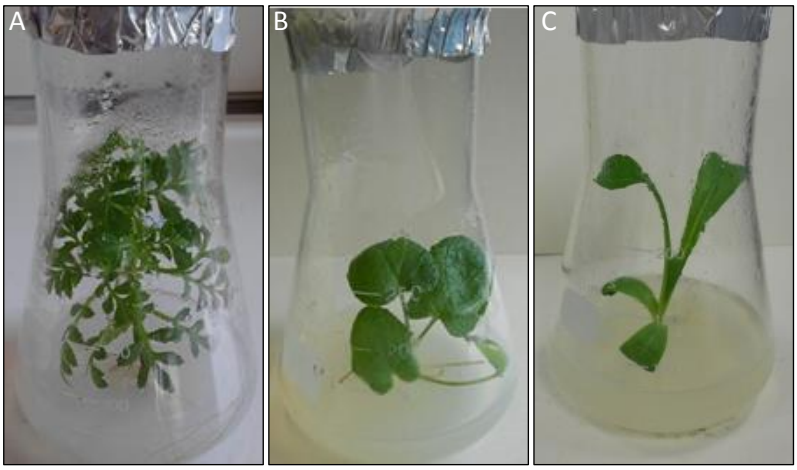


Fig. 2.3: *In vitro* culture of the Asteraceae plants. A: *T. patula*; B: *A. lappa*; C: *C. tictorius*.

2.2.4 Tissue culture

2.2.4.1 Callus culture

Leaves of sterile *in vitro* plants were used as explant for the induction of callus. The leaves were excised into pieces by a sterilized scalpel. The excised leaves were then transferred on solid MS medium supplemented with different combinations of auxin and cytokinin (Tab. 2.2). The cultures were maintained at 20°C in the dark or under a 16 h photoperiod. The effect of different combinations of phytohormones on callus induction was recorded after seven weeks of culturing. The cultures were transferred monthly into fresh medium.

Tab. 2.2: Combinations of phytohormones tested for induction of callus from leaf explants of Asteraceae plants. Abbreviation: NAA, Naphthalene acetic acid; 2,4-D, 2,4-Dichlorophenoxyacetic acid; BA, 6-Benzylaminopurine; IAA, Indole-3-acetic acid.

T. patula	*A. lappa*	*C. tinctorius*
0.01 mg/l NAA 0.45 mg/l kinetin 1.00 mg/l 2,4-D	2.00 mg/l 2,4-D 2.00 mg/l kinetin	2.00 mg/l 2,4-D 2.00 mg/l kinetin
		2.00 mg/l 2,4-D 2.00 mg/l BA
2.00 mg/l 2,4-D 2.00 mg/l kinetin	2.00 mg/l 2,4-D 2.00 mg/l BA	2.00 mg/l IAA 0.20 mg/l kinetin
		2.00 mg/l NAA 0.20 mg/l kinetin

2.2.4.2 Callus suspension culture

For initiation of cell suspension culture, about 2 g fresh-weight (FW) of greenish healthy callus were transferred to a 25 ml Erlenmeyer flask containing 10 ml MS medium supplemented with the same phytohormones as used for callus culture. The homogenization of the suspension culture was achieved on the rotatory shaker (120 rpm) in the dark at 20°C. After 7 days, 10 ml medium was added to the dense grown culture. At the end, the cultures were transferred to 250 ml flasks containing 50 ml of the same medium. Fourteen-day-old suspension culture cells were transferred into fresh medium by inoculating 2 g FW cells into 50 ml medium.

2.2.4.3 Root culture

Excised roots (approximately 1 cm long pieces) of aseptically growing seedlings were transferred to 10 ml MS medium in 25 ml Erlenmeyer flasks. The root cultures were kept on a rotary shaker (120 rpm) at 20°C in the dark and three-week-old cultures were transferred into fresh medium. For *A. lappa* and *C. tinctorius*, addition of different phytohormone combination was tested to establish the root culture (Tab. 2.3). In *C. tinctorius*, MS medium with 5 or 8 % (w/v) sucrose was also tested and the cultures were kept on a rotatory shaker. Other roots were cultivated statically (without stirring/shaking) in MS medium by using a very small volume of medium (6 ml) in a 250 ml flask (Bernard et al., 2011).

Tab. 2.3: Combinations of phytohormones for establishing root cultures of *A. lappa* and *C. tinctorius*. Abbreviation: NAA, Naphthalene acetic acid; IBA, Indole-3-butyric acid.

A. lappa	*C. tinctorius*
1 mg/l NAA+ 1mg/l IBA	1 mg/l NAA+ 1 mg/l IBA
1 mg/l NAA	1 mg/l NAA
0.2 mg/l NAA	0.2 mg/l NAA

2.2.4.4 Hairy root culture

Preparation of *Agrobacterium rhizogenes* and their growth condition for hairy root induction

A. rhizogenes strain LBA 1334 (Offringa et al., 1986) and *A. rhizogenes* DSMZ 3020 (DSMZ, Germany) were used. The bacterial stock culture kept at -80°C was spread by using a sterile loop on YMA medium (Tab. 2.4) supplemented with 50 µg/ml rifampicin. Plates were incubated in the dark at room temperature for 24 h and further maintained at 4°C in a refrigerator for one month. A single bacterial colony was picked, transferred to 10 ml YMB medium (Tab. 2.4) containing 50 µg/ml rifampicin and left to grow for 16 h in a shaker with constant agitation (150 rpm) at 26°C till the optical density at 600 nm was between 0.6-0.8. The bacterial suspension was centrifuged at 6000 rpm for 10 min, the supernatant was discarded and the resulting pellet was re-suspended in 1000 µl of MS medium supplemented with 0.4 mg/l naphthalene acetic acid (NAA). Freshly prepared acetosyringone (10 mg/ml in ethanol) was added to the bacterial suspension in a final concentration of 100 µM. The culture was left for one hour at room temperature before its use in transformation.

Tab. 2.4: Composition of YMB and YMA medium.

Medium	Compositions
Yeast Mannitol Broth (YMB)	KH_2PO_4 0.5 g/l $MgSO_4 . 7H_2O$ 0.2 g/l NaCl 0.1 g/l Yeast Extract 0.4 g/l Manitol 10 g/l ➢ pH-adjusted to 7 with 0.5 N NaOH, autoclaved.
Yeast Mannitol agar (YMA)	YMB with 1.5 % (w/v) agar. ➢ Agar added prior to autoclaving.

Transformation

Leaves of aseptically grown plants were used as explant for the transformation. Explants were prickled at the midrib with a sterile needle dipped in bacterial suspension. Explants prickled with a sterile needle dipped in sterile water served as control. All the infected and control explants were cultured on plates containing solid MS medium and incubated in the dark. After three days, the explants were transferred to 250 ml flasks containing 50 ml solid Gamborg's (B5) medium (Gamborg et al., 1968) (Tab. 2.5) supplemented with 250 µg/ml cefotaxime to kill the bacteria. After approximately two weeks, hairy roots were observed at the site of infection. These roots were allowed to grow until they were 6-8 cm long.

Tab. 2.5: Composition of Gamborg's (B5) medium.

Medium	Compositions	Per 1 l
Gamborg's (B5) medium	**1. Macro elements**	
	KNO_3	3000 mg
	$CaCl_2 . 2H_2O$	150 mg
	$MgSO_4 . 7H_2O$	500 mg
	$(NH_4)_2SO_4$	134 mg
	2. **Micro elements**	
	$MnSO_4 . 4H_2O$	10 mg
	H_3BO_3	3 mg
	$ZnSO_4 . 7H_2O$	2 mg
	$Na_2 MoO_4 . 2H_2O$	0.25 mg
	$CuSO_4 . 5H_2O$	0.025 mg
	$CoCl_2 . 6H_2O$	0.025 mg
	KI	0.75 mg
	$FeSO_4 . 7H_2O$	27.8 mg
	$NaH_2PO_4 . H_2O$	150 mg
	Na_2 EDTA	37.3 mg
	3. Vitamins	
	Nicotinic acid	1 mg
	Pyridoxin HCl	1 mg
	Thiamin HCl	10 mg
	Myo-Inositol	100 mg
	4. carbohydrates	
	Sucrose	20 g

	➢ pH-adjusted to 5.8 with 0.5 N NaOH, autoclaved.	
Gamborg's (B5) solid medium	Gamborg's (B5) medium with 0.8 % (w/v) agar. ➢ Agar added prior to auto-claving.	

Maintenance of hairy root cultures

The hairy roots emerging from infected explants (6-8 cm long) were excised from the mother tissue and transferred to B5 medium supplemented with 250 µg/ml cefotaxime and incubated on a rotary shaker under a constant agitation (120 rpm) in the dark at 20°C. Hairy roots (21-day-old) were transferred into fresh medium containing cefotaxime. Cefotaxime was completely omitted from the medium after five rounds of subculture with decreasing cefotaxime concentrations. For longer storage, bacteria-free hairy roots can be maintained in solid B5 medium. Two of the best growing hairy root clones (line 2, line 3) were selected for further experiments.

Confirmation of transformation by polymerase chain reaction (PCR)

Integration of the T-DNA with the *rolB* gene into the plant genome was confirmed by PCR amplification from genomic DNA. Genomic DNA was isolated from the two hairy root lines and from a normal root culture by using DNA purification Mini kit (Fermentas) as described in the manufacturer's guidelines. In both cases 100 mg fresh weight of plant material were used for genomic DNA isolation. The extracted genomic DNA was stored at -20°C for further use. PCR was done with a pair of gene-specific primers listed in the appendix (Tab. 7.1) to amplify a 862 bp *rol B* gene fragment. PCR was done in a total volume of 25 µl composed as follows:

DNA	1.5 µl
Forward primer (10 pmol/µl)	1 µl
Reverse primer (10 pmol/µl)	1 µl
10x reaction buffer with $MgCl_2$	2.5 µl
dNTPs (10 mM)	1 µl
Taq DNA polymerase (5 U/µl)	0.2 µl
Water up to	25 µl

The temperature program in TProfessional Thermocycler from Biometra was as follows:

Step	Temperature	Time
1	94°C	5 min
2	94°C	30 sec
3	55°C	1 min
4	72°C	1.5 min
5	72°C	10 min
6	10°C	Pause

The steps 2 to 4 were repeated 30 times before starting step 5.

DNA samples were analyzed by gel electrophoresis on 1 % (w/v) agarose gel stained with ethidium bromide. Plasmid DNA isolated from *A. rhizogenes* (LBA 1334) was used as positive control. This DNA was isolated according to (Sambrook et al., 1989) as described in 2.3.9.5 .

2.2.5 Elicitation treatment

2.2.5.1 Elicitors

Different elicitors were used for induction of polyacetylenes in the tissue culture. Details of elicitor preparation are given in Tab. 2.6.

Tab. 2.6: Elicitors used for induction of polyacetylenes in the tissue culture.

Elicitors	Supplier	Preparation and storage	Final concentration tested
Methyl-jasmonate (MeJ)	Aldrich	11 µl MeJ was dissolved in 989 µl 95 % (v/v) ethanol. The solution was freshly prepared before each experiment.	20, 40, 80, 100, 150, 200, 400 µM
Chitosan	Roth	Chitosan stock solution was prepared according to (Pitta-Alvarez and Giulietti, 1999). A concentrated chitosan solution was prepared by dissolving 125 mg chitosan in 1 % (v/v) acetic acid; the final volume was made up to 100 ml. The pH was adjusted to 5.5 with 1 N NaOH, sterilized by autoclaving at 120°C for 20 min, and kept at 4°C.	25, 75, 100, 300 mg/l

Vanadium (IV) oxide sulfate hydrate	Aldrich	Stock solution of 10 mg/ml was prepared in deionized water and filter sterilized by 0.20 µm filter. The stock solution was diluted 10 times when the lower concentration was required. The solution was freshly prepared before each experiment.	1, 10, 30, 50, 100, 200 mg/l
Copper sulfate	Merck		1, 10, 30, 50, 100 mg/l

2.2.5.2 Elicitor treatment of suspension cultures

To test the effect of elicitors, approximately 2 g FW of culture was transferred into 250 ml Erlenmeyer flask containing 50 ml MS medium supplemented with appropriate phytohormones and allowed to grow on a rotary shaker under a constant agitation (120 rpm) in the dark at 20°C. For *A. lappa*, the different elicitors were added individually to sixteen-day-old cultures (in the linear growth phase) while *C. tinctorius* cultures were elicited when they were seven days old (in the linear growth phase). The cultures were harvested after 48 h or 72 h depending on the elicitor. Control cultures were treated with ethanol in the case of MeJ and sterile water with the others. Duplicate flasks were used in each experiment.

2.2.5.3 Elicitor treatment of root cultures

The root culture of *A. lappa* was the only root culture tested for elicitation. Based on the results obtained with the suspension culture of *A. lappa*, 100 µM MeJ and 100 mg/l chitosan, were tested with root cultures. The elicitors were added individually to fifteen-day-old cultures. The cultures were harvested after 72 h of elicitation. Control cultures were treated with ethanol (MeJ) or sterile water (chitosan).

2.2.5.4 Elicitor treatment of hairy root cultures

To test the effect of elicitors on hairy root cultures of *T. patula*, approximately 1 g hairy roots were transferred into 250 ml Erlenmeyer flask containing 50 ml B5 medium and allowed to grow on a rotary shaker under a constant agitation (100 rpm) in the dark at 20°C. Two lines (lines 2 and 3) were used to optimize conditions for elicitation. In order to study the effect of type and concentration of elicitors on the production of thiophenes, the two lines were elicited with MeJ, chitosan and vanadyl sulfate across a range of concentrations (Tab. 2.6). The elicitors were added to thirteen-day-old cultures corresponding to linear growth phase of line 2 and the end of linear growth phase of line 3. Cultures were exposed to MeJ and chitosan for 72 h and to vanadyl sulfate for 48 h. Three individual cultures were used for each experiment. Control cultures were treated with sterile water in the case of chitosan and vanadyl sulfate or with ethanol in case of MeJ. The effect of culture age was studied by

adding an optimal concentration of the best elicitor to the culture of different ages, at the beginning, middle, and at the end of the stationary phase. The cultures were harvested after 72 h in the case of line 2 and after 48 h in case of line 3. Three individual cultures were used for each experiment. In order to study the effect of elicitor contact period, the two hairy root lines were treated with the optimum concentration of the best elicitor at the best age of the culture and then the cultures were harvested at different time points after addition of elicitor. Triplicate flasks were used in all experiments.

2.2.6 Phytochemical analysis of polyacetylene content

2.2.6.1 Extraction of polyacetylenes

Plant material was shock-frozen in liquid nitrogen and stored at -20°C or directly lyophilized. Lyophilized plant material was ground to a fine powder with a coffee mill prior to extraction. The extraction was performed according to the method of (Margl et al., 2002) with modifications and carried out at room temperature under dimmed room light. 1 g powdered plant material was extracted in 50 ml of 70 % (v/v) methanol for 25 min by using an ultrasonic bath. The internal standard (Tab. 2.7) was added before extraction. The extract was centrifuged at 4000 rpm for 20 min. Extraction was repeated two more times. The combined crude extract was purified three times by partitioning with 120 ml 1:1 (v/v) hexane/tert-butyl methyl ether (Hex/TBME). The organic phases were combined and evaporated to dryness. The residue was dissolved in 1 ml chloroform and filtered through a 0.2 µm syringe filter for subsequent GC analyses. The procedure was adjusted to smaller samples (0.1 g powdered of lyophilized culture tissue) by reducing the volume of solvent correspondingly. The medium (50 ml) from tissue culture was extracted twice with 50 ml ethyl acetate. The combined organic phases were dried over anhydrous sodium sulfate and concentrated to dryness. The residue was dissolved in 1 ml chloroform, filtered through a 0.2 µm syringe filter and analyzed by GC.

2.2.6.2 Identification of polyacetylenes

The polyacetylenes were identified by GC-MS. GC-MS analysis was carried out by using an Agilent 6890 N series gas chromatograph equipped with a ZB-5MS column (30 m × 0.25 mm × 0.25 µm (Phenomenex,USA)) and coupled with an 5975 B inert XL EI / CI mass spectrometer (Agilent). Injector and transfer line were set at 250°C. The temperature program was 50°C for 3 min, linear increase of temperature from 50°C to 310°C over 26 min (10°C/ min) and 310°C hold for 3 min. The split ratio was 1:10 and the injection volume 1 µl. Helium was used as a carrier gas with a flow rate of 1 ml/min. Identification of polyacetylenes was done by comparing the mass spectra with those found in the literature (Takasugi et al., 1987;

Binder et al., 1990b; Margl et al., 2002; Szarka et al., 2006). This method was chosen because authentic standards for the polyacetylens were not available. The retention index (RI) was calculated to help identifying the compounds. The RI was calculated using a set of hydrocarbons (even numbered from C_{16} to C_{26}). The alkane mixture was injected under the above mentioned temperature program before injection of polyacetylene containing samples. RI was calculated by linear interpolation: RI = 100 × y + 100 × (z-y) × (ta-ty/tz-ty); y = Carbon chain length of the shorter alkane, z = Carbon chain length of the longer alkane, ta = Retention time of the analyte, ty = Retention time of the shorter alkane, tz = Retention time of the longer alkane. All experiments were carried out at least in triplicate.

2.2.6.3 Quantification of polyacetylenes

The polyacetylenes were quantified by GC with flame ionization detection (FID). GC-FID analysis was achieved by using an Agilent 6890 N series gas chromatograph equipped with an HP-5MS column (30 m × 0.25 mm × 0.25 µm; Wicom). Injector and transfer line were set at 250°C. The temperature program was 50°C for 3 min, linear increase from 50°C to 310 over 26 min. The split ratio was 1:10 and the injection volume 1µl. Helium was used as a carrier gas with a flow rate of 1 ml/min. Quantification of polyacetylenes by GC-FID was done based on the peak area of an internal standard relative to the peak area of the compound. Internal standards are given in Tab. 2.7. The internal standard of known concentration was added during the extraction. Due to the lack of commercially available polyacetylene standards, the polyacetylenes were identified in GC-FID by the help of the RI (2.2.6.2). The RI from GC-MS (2.2.6.2) was compared to that obtained from GC-FID. RI was regarded as equal in GC-MS and GC-FID if they differed by less than 5.

Tab. 2.7: Compounds used as an internal standard for quantification of polyacetylenes.

Internal standard	Supplier	Preparation and storage
5-Bromo-2,2'-bithiophene	Aldrich®	0.05 g/10 ml methanol stored at -20°C.
1-Tridecene	Alfa Aesar®	
2-Bromothiophene	Aldrich®	
4-Chlorobenzophenone	Aldrich®	

2.3 Material and methods for carrot cell suspension culture as transgenic production platform

2.3.1 Bacterial strains, plasmid vectors, and PCR primers

Bacterial strains

***E. coli*-strain XL1-Blue**

This Bacterial strain with the genotype *recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* [*F´ proAB lacI*^{q} *ZΔM15 Tn10* (Tet^{r})] was ordered from Stratagene (Agilent Technologies).

***E. coli*-strain DH5α**

This bacterial strain with the genotype [F' *φ80δlacZ9M15 end A1hsdR17*(*rk*$^{-}$ *mk*$^{+}$) *supE44thi-1 λ*$^{-}$*gyrA96 relA19*(*lacZYA-argFV169*) *deoR*] was ordered from Invitrogen (Life Technologies™).

***Agrobacterium tumefaciens* C58C1**

This bacterial strain harbors the nopaline disarmed Ti plasmid pMP90 (pTiC58DT-DNA) carrying a gentamycin resistance gene. The marker gene present in the genome is the rifampicin resistance gene.

Plasmid vectors

pRT101

The vector pRT101 (Töpfer et al., 1987) contains the strong, nominally constitutive, 35S promoter from cauliflower mosaic virus (CaMV35S) and an ampicillin resistance gene for bacterial selection. The size of the vector is 3381 bp. It is used to provide the CaMV35S promoter to the cDNA before cloning into pPZP111 expression vector.

pPZP111

pPZP111 is a binary vector with a size of 8910 bp (Hajdukiewicz et al., 1994). It contains a chloramphenicol resistance gene as selectable marker for growth in *E. coli* and *A. tumefaciens* and a kanamycin resistance gene as selectable marker for plants.

pPZP221

pPZP221 is a binary vector with a size of 8732 pb (Hajdukiewicz et al., 1994). It contains a streptomycin resistance gene as selectable marker for growth in *E. coli* and *A. tumefacien* and a gentamycin resistance gene as selectable marker for plants.

Binary vector pCAMBIA1302

pCAMBIA1302 is a binary vector with a size of 10.550 bp (www.cambia.org). The T-DNA region contains the CaMV35S promoter upstream of the green fluorescent protein (*GFP*) gene as well as the gene encoding hygromycin phosphotransferase (*hpt*) that confers

hygromycin resistance for plant selection. Additionally, it contains a kanamycin resistance gene (*nptII*) for selection in *E. coli* and *A. tumefaciens*.

PCR primers

The gene specific oligonucleotides listed in the appendix (Tab. 7.1) were designed to have a length of 18-25 nucleotides, a GC content of about 50 % and a melting temperature between 50°C-65°C. The lyophilized oligonucleotides were dissolved to a concentration of 100 µM in water and stored at -20°C. Working solutions of 10 µM solutions were prepared and also stored at -20°C.

2.3.2 Cultivation of carrot callus suspension culture

A carrot callus suspension culture was donated by Marion Wiggermann (Wittstock lab). It was derived from callus that originated from leaf, stem and root of an aseptically grown plant and was maintained in AM4/1 medium (Tab. 2.8). The cultures were incubated at 20°C in the dark with shaking at 120 rpm. Every 10 days, 2 g FW cells were used to inoculate 50 ml fresh medium in a 250 ml flask.

Tab. 2.8: Composition of AM4/1 medium.

Medium	Compositions	Per 1 l
AM4/1 medium	**1. Macro elements**	
	KNO_3	620 mg
	$CaCl_2 . 2H_2O$	440 mg
	$MgSO_4 . 7H_2O$	370 mg
	KNO_3	2500 mg
	KH_2PO_4	400 mg
	Fe EDTA	80 mg
	2. Micro elements	
	$MnSO_4 . 4H_2O$	10 mg
	H_3BO_3	3 mg
	$ZnSO_4 . 7H_2O$	2 mg
	$Na_2 MoO_4 . 2H_2O$	0.25 mg
	$CuSO_4 . 5H_2O$	0.025 mg
	$CoCl_2 . 6H_2O$	0.025 mg
	KI	0.75 mg
	3. Vitamins	
	Nicotinic acid	1 mg
	Pyridoxin HCl	1 mg
	Thiamin HCl	10 mg

	Myo-Inositol	100 mg
	4. Carbohydrates	
	Sucrose	40 g
	5. Hormones	
	NAA (0.5 mg/ml) in absolute ethanol.	0.02 ml
	Kinetin (0.5 mg/ml) dissolved in little 0.5 M HCl, slightly warm, then distilled water was added.	0.9 ml
	2,4 D (1mg/ml) in absolute ethanol	1 ml
	➢ pH-adjusted to 5.8 with 0.5 N NaOH, autoclaved.	
solid AM4/1 medium	AM4/1 with 0.9 % (w/v) agar ➢ Agar added prior to autoclaving.	

2.3.3 Synchronization of carrot callus suspension culture

The synchronization of carrot callus suspension culture basically relies on an cell cycle arrest and release strategy using fluorodesoxyuridine (FDU)/thymidine as described earlier (Imani et al., 2002). FDU reduces the rate of DNA synthesis and arrests the cells at the G1/S boundary. For synchronization of the carrot callus suspension culture, 0.1 µM FDU (Acros, Belgium) was added to the medium 24 h before transformation. After 24 h, the cells were transferred to fresh medium supplemented with 10 µM thymidine (Sigma-Aldrich, USA) to initiate the transition of the synchronized cultures from late G1-phase into the S-phase for DNA replication. For transformation, *A. tumefaciens* C58C1 carrying the expression construct was added simultaneously with thymidine.

2.3.4 *Agrobacterium*-mediated transformation of carrot suspension culture

2.3.4.1 Method I

This method was similar to (Hardegger and Sturm, 1998) and relied on *A. tumefaciens*-mediated transformation of a suspension culture without conversion to a callus. *A. tumefaciene* C58C1 carrying the desired construct was plated on solid YMB medium (2.2.4.4) (Tab. 2.4) containing 100 µg/ml rifampicin and the plasmid-specific antibiotic. The plate was incubated at 28°C for 2-3 days. One colony was inoculated into 10 ml YEP medium (see be-

low) supplemented with the same antibiotics and incubated overnight at 28°C with shaking at 220 rpm. Subsequently, 1.5 or 2 ml culture were transferred to 500 ml Erlenmeyer flask containing 100 ml of YEP medium supplemented with the same antibiotics and the culture was maintained under the same conditions. When the bacterial culture reached an optical density (OD_{600}) between 0.8-1, the bacterial suspension was collected by centrifugation at 4000 rpm for 20 min. The bacterial pellet was gently re-suspended in AM4/1 medium (2.3.2) (Tab. 2.5) and centrifuged again. A pellet of about 200 µl was re-suspended in 2 ml AM4/1 medium. Freshly prepared acetosyringone (0.05 M in ethanol) was added to the bacterial suspension to a final concentration of 100 µM and allowed to stand for 1.5 h at room temperature before transformation of plant cells.

YEP medium	10 g/l Bactopeptone
	10 g/l Yeast extract
	5 g/l Sodium chloride, autoclaved

The synchronized and non-synchronized carrot cells were collected by centrifugation at 1000 rpm for 2 min. The cell pellet was transferred to 250 ml Erlenmeyer flasks containing 50 ml of the corresponding carrot callus suspension culture medium and 500 µl bacterial suspensions were added. The cells were co-cultivated at 22°C with shaking at 110 rpm. After 3 days, the cells were collected in a Falcon tube and centrifuged at 1000 rpm for 2 min to separate the carrot cells from the majority of bacterial cells. The plant cells were transferred into 50 ml fresh medium containing 250 µg/ml cefotaxime to stop bacterial growth and with addition of selective antibiotic for transformed plant cells. The cultures were incubated again for a few days, but checked daily. The cultures were passed into new medium containing cefotaxime and the selective antibiotic weekly. Cells without addition of *A. tumefaciens* were used as a control.

The protocol was tested first by transformation with pCAMBIA1302 for expression of *GFP*. After synchronized or non-synchronized cells were supplemented with pCAMBIA1302, the cells were incubated for three days at 22°C in a rotatory shaker at 110 rpm. Daily, 500 µl of each culture were centrifuged (5 min at 6,000 rpm). The pellet was checked under UV light for *GFP* fluorescence. As observation of fluorescence may be hindered by the cell wall, fluorescence was checked in extracts of transformed cells. After three days of co-cultivation, 250 µg/ml cefotaxime and 30 µg/ml hygromycin were added. The cells were harvested at 8, 12, 18 and 25 days. 1 g FW cells were mixed with seasand (50 % w/w) and homogenized in 50 mM MES buffer (pH 6.0) in a mortar on ice for 15 min. Cell homogenates were centrifuged at

14,000 rpm for 20 min. Denatured and non-denatured samples were subjected to SDS-PAGE (2.3.10.1) and gels monitored under UV light followed by Coomassie blue staining for documentation.

2.3.4.2 Method II

This method was derived from (Iantcheva et al., 2014) and relied on *A. tumefaciens* mediated transformation of suspension cultures and subsequent generation of callus for selection of transgenic lines. *A. tumefaciene* C58C1 carrying the desired construct was prepared as described in 2.3.4.1 with some modifications. When the bacterial culture reached an OD_{600} between 0.8-1, 10 ml of bacterial suspension were transferred to 50 ml Falcon tube and centrifuged at 4000 rpm for 20 min. The bacterial pellet was washed with AM4/1 medium and centrifuged again. The pellet was dissolved in 25 ml AM4/1 medium when the OD_{600} required for the transformation was 0.3, while dissolved in 10 ml, if the OD_{600} required was 0.8. Freshly prepared acetosyringone was added to the bacterial suspension in the desired concentration (25, 50 or 100 µM) and the suspension incubated at room temperature for 1.5 h before transformation.

For preparation of carrot suspension culture cells, cells (10 days after subculture) were collected in 50 ml Falcon tube and centrifuged at 1000 rpm for 2 min. The cell pellet was transferred into 50 ml fresh medium and 6 ml of cell suspension was transferred into 50 ml Falcon tube. 300 µl of bacterial suspension was added and the suspension incubated at 22°C with shaking at 110 rpm in the dark. After 2 or 3 days of inoculation, the cells were sedimented by centrifuged for 3 min with 100 × g at room temperature. The cell pellet was washed with 10 ml AM4/1 medium. After three cycles of sedimentation and washing, the cells were resuspended in an equal volume of medium by shaking by hand. Droplets of 20 µl suspension were spotted on solid AM4/1 medium containing 250 µg/ml cefotaxime and the plasmid-specific antibiotic. The plates were incubated at 20°C at 16 h light. The cultivation continued until sufficient callus was observed. The amount of cefotaxime was gradually decreased until no growth of bacteria was observed. To gain large amount of material, the callus was converted to suspension culture by transferring the callus into 30 ml AM4/1 medium in 100 ml Erlenmeyer flask supplemented with cefotaxime if required and the selective antibiotics. The cells were then transferred into 50 ml of the same medium with addition of the selective antibiotic.

The method was tested with *A. tumefaciens* C58C1 carrying pCAMBIA1302. The following parameters were varied to identify most efficient transformation conditions: OD_{600} of bacterial suspension (0.3, 0.8), synchronized or non-synchronized plant cells, co-cultivation of plant cells with *Agrobacteria* (static, agitated, 2 or 3 days), concentration of acetosyringone

(25, 50, 100 µM). Transformation efficiency was calculated as the percentage of antibiotic-resistant calli of the total number of droplets plated on selective medium.

2.3.5 Isolation of genomic DNA from transformed carrot cells

Cells were harvested from suspension cultures. 100 mg FW of transformed cells and non-transformed cells were ground to a fine powder in liquid nitrogen using pre-chilled mortar and pestle. The powdered cells were transferred to 2 ml Eppendorf tube and 500 µl SDS buffer (see below) was added with gentle mixing by inverting the Eppendorf tube 4-6 times. 10 µl (10 mg/ml RNaseA) was added with shaking by inverting the tube. The cells were incubated at 37°C for 15 min followed by incubation at 65°C for 10 min. 75 µl 5 M potassium acetate was added and the samples were incubated on ice for 20 min followed by addition of 600 µl phenol:chloroform (1:1) and thorough mixing. Cell debris and precipitates were removed by centrifugation at 4°C and 12,000 rpm for 10 min. The aqueous layer was transferred into a new 2 ml Eppendorf tube, an equal volume of chloroform was added and samples mixed well. The mixture was centrifuged at 4°C and 12,000 rpm for 10 min. After centrifugation, the aqueous layer was transferred into a new 1.5 ml Eppendorf tube and the DNA was precipitated with double volume of 100 % EtOH. After incubation overnight at -20°C the mixture was centrifuged as above. The pellet was washed with 80 % EtOH and air-dried. DNA was eluted with 30 µl water and PCR (2.3.9.1) with specific oligonucleotides was performed to check for the presence of the integrated gene in the transformed lines. Genomic DNA was stored at -20°C.

SDS buffer	50 mM Tris, pH 8
	10 mM EDTA, pH 8
	100 mM NaCl
	1 % (w/v) SDS

2.3.6 Cryopreservation of suspension-cultured cells and transgenic cell lines

The cryopreservation of carrot suspension culture was done by a classical cryopreservation technique. This technique is based on dehydration of plant material before storage in liquid nitrogen at -196°C. The protocol was carried out according to (Menges and Murray, 2004) with some modifications. The procedure depends on DMSO and sorbitol as cryoprotectant solution. 5 ml of ten-day-old suspension culture cells were pre-cultured in 50 ml AM4/1 medium (Tab. 2.8) at 20°C with shaking at 120 rpm. After 3 days of incubation, cell suspensions were transferred into 50 ml falcon tube and cells were harvested by centrifugation at 387 x g

for 3 min. The weight of the cell pellet was determined and an appropriate volume of the same medium containing 0.5 M sorbitol was added to reach a packed cell volume (PCV) of approximately 40 %. Re-suspended cells were transferred into a 100 ml Erlenmeyer flask and incubated for further 2 days under the same conditions. Cell suspensions were then pre-cooled for 25 min at 4°C with rotation at 140 rpm in the dark. An appropriate volume of pre-cooled DMSO was added to reach a concentration of 5 % DMSO. Incubated was continued for further 1 h at 4°C with rotation at 140 rpm. 1.8 ml cell suspension were transferred into 2 ml cryovials. The vials were placed into a freezing-container containing isopropyl alcohol and the container was stored in standard plastic cryo-box. The box was stored at -80°C or vials were plunged in liquid nitrogen after 4 h at -80°C and stored under liquid nitrogen. The protocol described by (Ogawa et al., 2012) with modification was also tested.

Thawing of cryopreserved cells should be as rapid as possible to avoid ice crystal formation (Mazur, 1984). For thawing of frozen cells, the cryovials were placed immediately from liquid nitrogen into a water bath at 40°C for 3 min. Thawed cells were dropped on a nylon membrane (70 µm pore size, Omnilab-Laborzentrum GmbH & Co. KG, Germany) which was placed on a plate containing solid AM4/1. Plates were sealed with parafilm and incubated for 3 h in the dark. The filter carrying the spread cell suspension was then transferred onto a fresh medium and incubated in the dark. After 7 days of growth, recovered cell callus material was re-suspended separately for each spot in 50 ml AM4/1 medium and incubated in the dark with shaking at 120 rpm.

2.3.7 Transformation of *A. tumefaciens* C58C1

Preparation of competent *A. tumefaciens* cells for electroporation

The frozen glycerol stock of bacterial cells from -80°C was plated onto YMB agar plates (Tab. 2.4) containing 100 µg/ml rifampicin and incubated overnight at 28°C. A single bacterial colony was inoculated in 10 ml YEP medium (2.3.4.1) containing 100 µg/ml rifampicin and cultured overnight at 28°C with shaking at 220 rpm. The overnight culture was transferred to 500 ml sterilized Erlenmeyer flask containing 290 ml YEP medium supplemented with 100 µg/ml rifampicin and incubated at the same condition for 3-7 h until the bacterial culture reached an OD_{600} between 0.5-0.7. The culture was chilled on ice and then centrifuged at 5,000 × g for 10 min. The culture medium was decanted and the pellet re-suspended in 300 ml ice-cold 10 % (v/v) glycerol. This washing step was repeated three more times with stepwise reduction of the volume for re-suspension to 150, 60 and 3 ml ice-cold 10 % (v/v) glycerol. The suspension was dispensed in 50 µl aliquots, frozen in liquid nitrogen and stored at -80°C until use.

Transformation of *A. tumefaciens* by electroporation

After thawing the frozen aliquot of *A. tumefaciens* C58C1 electrocompetent cells on ice, the desired plasmid was added to the bacteria and the sample mixed gently with a pipette. The mixture was transferred into a pre-cooled 2 mm electroporation cuvette (Bio-Rad laboratories). An electric pulse with 2.5 kV, 400 kΩ, 25 µF was applied, then immediately 1 ml YMB liquid medium was added. The mixture was transferred to 50 ml Falcon tube containing 9 ml YMB liquid medium and shaken at 28°C for 3 h. After this incubation period, different aliquots were streaked out on YMP agar plate containing 100 µg/ml rifampicin and the proper selection antibiotic. The plates were incubated at 28°C for three days. For storage of *A. tumefaciens*, 700 µl bacterial suspension from 10 ml overnight culture were mixed with 300 µl of sterile glycerol and stored at -80°C.

Isolation of DNA from transformed *A. tumefaciens*

Single colony of transformed *A. tumefaciens* C58C1 was used to inoculate 10 ml YEP medium (2.3.4.1) containing 100 µg/ml rifampicin and other appropriate antibiotics and incubated overnight at 28°C with shaking at 220 rpm. 4 ml of the overnight culture was centrifuged for 4 min at 6500 rpm. The pellet was washed twice with 500 µl 100 mM Tris-HCl, pH 8 by vortexing and centrifugation for 3 min at 6,500 rpm. The pellet was re-suspended in 600 µl Agrobacterium DNA extraction buffer (see below), 50 µl proteinase K (5 mg/ml) was added and the sample vortexed vigorously. After addition of 160 µl 10 % SDS, tubes were inverted 4-6 times and incubated at 65°C for 1 h. After cooling to room temperature, 500 µl phenol/chloroform (1:1) (Roth®) was added. The suspension was mixed by inverting the tube and centrifuged at 14,000 rpm for 25 min. The upper phase was transferred to a new 1.5 ml Eppendorf tube and 0.6 volumes of isopropanol were added. The sample was vigorously mixed and centrifuged at 14,000 rpm for 25 min. The pellet was washed thoroughly with 500 µl 70 % absolute ethanol and centrifuged for 10 min at 13,000 rpm. The pellet was dried and dissolved in 20 µl 10 mM Tris-HCl, pH 8. The isolated DNA was stored at -20°C until further use or used to transform *E. coli* DH5α for later plasmid isolation.

Agrobacterium DNA extraction buffer	110 mM Tris-HCl 55 mM EDTA 1.54 M NaCl 1.1 % CTAB ➢ pH-adjusted to 8, autoclaved

2.3.8 Transformation of *E. coli*

Preparation of competent *E. coli* cells for heat-shock transformation

Chemically competent cells were prepared by the calcium chloride method (Cohen et al., 1972; Dagert and Ehrlich, 1979). Cells from a frozen glycerol stock were plated onto solid LB medium (Lysogeny broth medium with agar, see below) without antibiotic addition and incubated overnight at 37°C. A single bacterial colony was used to inoculate 5 ml LB medium and cultured overnight at 37°C with shaking at 250 rpm. 500 µl of overnight culture was transferred to 50 ml LB medium in 250 ml sterilized Erlenmeyer flask. The cells were cultured at 37°C for 3-4 h until the bacterial culture reached OD_{600} between 0.4-0.5. The culture was transferred to an ice-cold 50 ml Falcon tube. After placing on ice for 10 min, the cells were centrifuged at 4°C and 3000 rpm for 10 min. The bacterial pellet was re-suspended carefully in 25 ml ice-cold 0.1 M $CaCl_2$. After 10 min incubation on ice, the cells were recovered by centrifugation at 4°C and 3000 rpm for 10 min. The pellet was gently dissolved in 5 ml 0.1 M $CaCl_2$ containing 15 % (v/v) glycerol. Then the cell suspension was kept at 4°C for about 24 h. 90 µl aliquots were dispensed into pre-chilled Eppendorf tubes, frozen immediately in liquid nitrogen and stored at -80°C.

LB-Medium	1 % (w/v) Tryptone/Peptone (Casein)
	0.5 % (w/v) Yeast extract
	0.17 M Sodium chloride
	For solid medium 1.5 % (w/v) agar were added
	➢ pH-adjusted to 7.5, autoclaved

Transformation of *E. coli* by heat-shock

An aliquot (90 µl) of chemical competent *E. coli* cells from a -80°C storage was thawed on ice. As soon as the competent cells were thawed, 150 ng of plasmid or 10 µl of ligation mixture were added and the mixture left for 25 min on ice. The cells were incubated for 45 sec at 42°C, followed by immediate incubation on ice for 5 min. The cells were grown in 250 µl SOC medium (Super optimal broth medium with addition of glucose, see below) by shaking for 1.5 h at 37°C. For selection of transformands, an aliquot (100 µl) of the cells was plated on solid LB medium containing the respective antibiotics and incubated overnight at 37°C. For storage, 750 µl bacterial suspension from a 5 ml overnight culture were mixed with 250 µl LB-glycerol (see below) and stored at -80°C.

SOC-Medium	2 % (w/v) Tryptone/Peptone (Casein) 0.5 % (w/v) Yeast extract 10 mM Sodium chloride 2.5 mM Potassium chloride pH-adjusted to 7.5, autoclaved and then add the sterile filtered solution of: 10 mM Magnesium sulfate 10 mM Magnesium chloride 22 mM Glucose

LB-Glycerol	1 g Tryptone/Peptone (Casein) 0.5 g Yeast extract 1 g Sodium chloride Fill up to 40 ml with water, pH-adjusted to 7.5 60 ml Glycerol was added, autoclaved

2.3.9 Molecular biology techniques

2.3.9.1 PCR

PCR is an *in vitro* method of nucleic acid synthesis, by which a particular segment of DNA can be specifically replicated by means of thermostable DNA-dependent polymerase and two oligonucleotides which bind upstream and downstream of the region to be amplified (Saiki et al., 1988). PCR was setup as described in 2.2.4.4. Reactions were performed in 200 µl PCR reaction tubes using the TProfessional Thermocycler from Biometra. The dream Taq buffer contains Mg^{2+} ions to reach a final concentration of 2 mM. Modifications were introduced depending on the Tm of oligonucleotides, the DNA fragment length, the abundance of the template, the desired level of accuracy, and the efficiency of the reaction. Taq-DNA polymerase has no 3´→ 5´ exonuclease activity to correct wrongly built in nucleotides (Tindall and Kunkel, 1988) and was therefore only used for analytical PCR. For the generation of the expression constructs and for other applications requiring a high accuracy of replication, the thermostable DNA Q5 polymerase (New England Biolabs Inc., Ipswich, USA) with 3´→ 5´ exonuclease activity, fused to a processivity-enhancing Sso7d domain to support DNA amplification was used.

2.3.9.2 Agarose gel electrophoresis and DNA purification from the gel

Agarose gels were prepared at concentrations of 1 % (w/v) agarose in Tris-boric acid-EDTA-(TBE)-buffer for most applications; 2 % (w/v) agarose was used for very small DNA molecules. The mixture was boiled in the microwave to dissolve the agarose. After cooling down to about 50°C, Midori green (Nippon Genetics Europe GmbH, Germany) was added to a final dilution of 1:30,000. Gel trays, combs, flow chamber and the power supply were purchased from Biorad. The solidified gel was immersed into 1x TBE buffer in a gel tank. The DNA samples were supplemented with 6x loading dye (Fermentas) and pipetted into the gel wells. As a molecular size marker generally a 1 kb DNA ladder from Fermentas was used. For separation of DNA fragments, 75 to 95 V were applied for about 30 to 60 min. DNA bands were visualized under a transilluminator (Vilber).

TBE-buffer	890 mM Tris
	890 mM Boric acid
	20 mM EDTA

For cloning of PCR products, the corresponding DNA bands of the right size were excised from the agarose gel on a UV-transilluminator (Nippon Genetics Europe GmbH) and extracted by using Qiaex® II Gel extraction kit (Qiagen GmbH) according to the manufacturer's instructions. The principle of this kit is based on the dissolving of the agarose, and the selective binding of the DNA-fragments to the QIAEX II silica gel particles in the presence of chaotropic salts.

2.3.9.3 DNA Digestion

Restriction enzyme digestions were carried out in the buffer supplied with the enzyme and in accordance with the supplier's recommendations for temperature and duration of the digestion. Restriction digests were setup as shown in Tab. 2.9 and Tab. 2.10.

Tab. 2.9: Composition of a typical restriction digest of PCR-product and corresponding vector with two enzymes.

Component	PCR product- restriction	pRT101 restriction
DNA	15.3 µl	8 µl (16 µg)
Buffer G	3 µl	2 µl
*BamH*I	0.5 µl	0.5 µl
*Xho*I	1 µl	1 µl
Water	10.2 µl	13.5 µl

Tab. 2.10: Composition of a typical restriction digest of plasmids with one enzyme.

Component	TGG1-pRT101 restriction	pPZP111 restriction
DNA	6 µl (4 µg)	2 µl (4 µg)
Buffer O	2 µl	2 µl
*Pst*I	0.5 µl	0.5 µl
Water	11.5 µl	15.5 µl

2.3.9.4 Ligation of DNA fragments

The ligation reaction was performed using the T4 DNA Ligase with the supplied buffer. A negative control reaction which contained all the components except the insert was done in parallel. The supplied buffer contained ATP, which is an essential cofactor of T4 DNA ligase. The reaction was usually carried out in 10 µl final volume using 1 µl of T4 DNA ligase, and an approximate molar vector:insert proportion of 1:3 and 1:6. The reactions were incubated overnight at 4°C. The whole ligation reaction was used to transform 90 µl competent *E.coli* XL1-blue as described in 2.2.8.

2.3.9.5 Isolation of plasmid DNA from *E. coli*

A culture (5 ml) of transformed bacteria grown in LB-medium with selective antibiotic was used for isolation of plasmid DNA according to (Sambrook et al., 1989). Briefly, a cell pellet obtained from 4 ml culture was re-suspended in 300 µl of ice-cold buffer P1 (see below) containing RNaseA (10 µl of 10 mg/ml RNaseA added per ml buffer, see below) and vortexed, 300 µl buffer P2 (see below) were added to lyse the cells and the bacterial suspension was gently mixed by inverting the tube 4-6 times and incubated at room temperature for 5 min. Precipitation of proteins and denaturation of large chromosomal DNA was done by adding 300 µl (ice-cold) buffer P3 (see below), inverting the tube 4-6 times and incubating on ice for 15-20 min. The mixture was centrifuged at 13,000 rpm for 10 min. The supernatant containing DNA (800 µl) was transferred into a new 2 ml Eppendorf tube and an equal volume of chloroform was added. The mixture was vortexed followed by centrifugation at 13,000 rpm for 10 min. The aqueous upper layer was transferred into a new 1.5 ml Eppendorf tube. Isopropanol (0.7 volumes) was added, the mixture was vortexed followed by centrifuged at 13,000 rpm for 20 min to precipitate plasmid DNA. The pellet was washed with 500 µl (70 % ethanol) before it was dried at 37°C for 15 min and dissolved in 50 µl of sterile distilled water. DNA content was determined spectrophotometrically. The plasmid was stored at -20°C for further use.

RNaseA	10 mg/ml in 0.01 M Sodium acetate (pH 5.2) Heat to 100°C for 15 min. After cooling to room temperature pH was adjusted with 0.1 volume of 1 M Tris-HCl (pH 7.4), stored at -20°C
P1-Buffer	50 mM Tris 10 mM Sodium-EDTA pH-adjusted to 8, autoclaved RNase A was added freshly before use
P2-Buffer	200 mM Sodium hydroxide 1 % (w/v) SDS Autoclaved
P3-Buffer	3 M Potassium acetate ➢ pH-adjusted to 5.5 with concentrated acetic acid, autoclaved

2.3.9.6 DNA-sequencing

For sequencing, 1.5 µg plasmid DNA dissolved in 15 µl of water were sent to Eurofins MWG Operon Company (Ebersberg, Germany).

2.3.10 Protein biochemical methods

2.3.10.1 SDS-PAGE

SDS-PAGE is a commonly used method for mostly analytical protein separation and molecular mass determination of protein subunits. The protein samples are denatured in loading buffer at 95°C. DTT in the loading buffer ensures reducing conditions. During denaturation, the protein is defolded and becomes uniformly coated with negative charges from SDS in the loading buffer. The gels were prepared by Loretta Heise (Wittstock lab) as described in Tab. 2.11. For electrophoretic separation, the protein samples were supplemented with the 3x loading buffer (see below), denatured for 5 min at 95°C, and loaded into the wells of the stacking gel. Electrophoresis was done in SDS-electrode buffer (see below) at 20 mA for about 20 min in which samples passed the stacking gel and at 30 mA for about 30 to 40 min in which samples passed the separating gel.

Tab. 2.11: Composition of SDS-PAGE. The stacking gel buffer was 0.5 M Tris buffer (pH 6.8), while the resolving gel buffer was 1.5 M Tris buffer (pH 8.8). The gels produced were approximately 83 x 70 x 0.75 mm and had ten slots for samples.

	Stacking gel	**Resolving gel 10.5 %**
Buffer	6.25 ml	16 ml
30 % Acrylamide-, Bisacrylamide solution 37.5:1	3.75 ml	22.4 ml
Water	15 ml	24.6 ml
10 % SDS	250 µl	640 µl
10 % Ammonium peroxydisulfate	75 µl	320 µl
N,N,N`,N`-tetramethylethylenediamine	25 µl	32 µl

SDS-electrode buffer	0.025 M Tris 0.19 M Glycin 3.5 mM SDS
3 × loading buffer	6.25 ml 0.5 M Tris-buffer pH 6.8 4.4 ml Water 5 ml Glycerin 1 g SDS 21 mg Bromphenolblue fresh DTT to 150 mM

After electrophoretic separation, proteins were visualized by staining with Coomassie Brilliant Blue. The gel was rinsed with water and shaken with the solutions described below: first, one hour in Coomassie staining solution, secondly 30 min in destaining solution I and then overnight in destaining solution II. After the gel was washed with water, it was scanned and dried between cellophane sheets for storage.

Coomassie staining solution	50 % (v/v) Ethanol 0.5 % (w/v) Coomassie-Brilliant-Blue R250 10 % (v/v) Acetic acid
Destaining solution I	50 % (v/v) Ethanol 10 % (v/v) Acetic acid
Destaining solution II	16.5 % (v/v) Ethanol 5 % (v/v) Acetic acid

2.3.10.2 Western blot

Proteins were separated by SDS-PAGE, and the gel was washed with water and incubated for 15 min in Tris/CAPS (N-cyclohexyl-3-aminopropanesulfonic acid). One nitrocellulose membrane (Protran BA 85, GE Healthcare) with 0.45 µm pore diameter and extra thick blot papers (BioRad Laboratories GmbH, Deutschland) were cut to a size of 6 × 9 cm. The membrane and two extra thick blot papers were equilibrated for 30 min in anode buffer. The two extra thick blot papers, the nitrocellulose membrane, and the gel were arranged in the blotting cell and covered by two extra thick blot papers soaked in cathode buffer. The transfer was performed for 12 minutes with 22 V. Then the membrane was rinsed with water and TTBS (Tris-buffered saline with Tween 20, see below) and incubated for 1 h at room temperature with shaking in 3 % BSA in TTBS. After 1 h the blocked nitrocellulose membrane was incubated with primary antibody (Anti-His serum (Roth®), 1:5000 in TTBS with 3 % BSA) overnight at 4°C. The membrane was washed 3 times with TTBS buffer for 10 min, once with TBS (Tris-buffered saline, see below) for 5 min. The membrane with primary antibody was incubated in a 1:10,000 dilution of secondary antibody (anti-rabbit IgG (Sigma) in TTBS with 3% BSA). After 2 h of incubation, the membrane was washed again 3 times for 10 min with TTBS, and once with TBS for 5 min. The membrane with second antibody was washed once for 5 min with alkalin phosphatase (AP) buffer (see below) and then incubated in 15 ml AP-buffer supplemented with 100 µl nitrobluetetrazolium chloride (50 mg/ml in 70 % dimethylformamid (DMF)) and 50 µl 5-bromo-4-chloro-3-indolyl phosphate (Applichem, 50 mg/ml in DMF) from 3 to 15 min until bands became visible. The reaction was stopped by washing in water. The dried membrane was stored in the dark.

Anode buffer	0.06 M Tris 0.04 M CAPS pH 9.6 15 % (v/v) Methanol
TBS	20 mM Tris HCl pH 7.5 150 mM NaCl
Cathode buffer	0.06 M Tris 0.04 M CAPS pH 9.6 0.1 % (w/v) SDS
TTBS	TBS 0.1 % (v/v) Tween 20
Tris/CAPS	0.06 M Tris 0.04 M CAPS pH 9.6
AP-buffer	100 mM Tris pH 9.5 100 mM NaCl 5 mM $MgCl_2$

2.3.10.3 Plant extracts for detection of myrosinase

Crude extracts of transformed carrot suspension culture cells were prepared by mixing the cells with seasand (50 % w/w) and grinding in a mortar on ice with 50 mM MES buffer, pH 6.0 (1 ml of buffer per gram of cells). Cell homogenates were centrifuged at 9,000 rpm for 25 min. The supernatants were kept on ice before use for measurements of myrosinase activities. The protein content of extracts was determined photometrically by using the Pierce BCA™ Protein Assay Kit (Thermo Fisher Scientific, Germany). To create a calibration line, BSA was measured in parallel in concentrations from 0-2 mg/ml in the same buffer as the sample. The colorimetric determination was carried out at 562 nm after 30 min incubation at 37°C in microtiter plate by using the microplate reader Sunrise^{TM}.

2.3.10.4 Myrosinase assay

The myrosinase assay was carried out in a total volume of 500 µl. Different volumes of crude plant extract (50, 100 and 200 µl) were incubated with 2 mM allylglucosinolate in 50 mM MES buffer (pH 6.0). The mixtures were incubated 40 min at 23°C before 50 µl of phenylcyanide (1:10,000 in MeOH) were added as an internal standard. The mixtures were extracted twice with approximately 750 µl dichloromethane. The dichloromethane phases were combined and passed through sodium sulfate to get rid of water. The negative control was carried out without the addition of crude extract. The positive control was carried out with addition of myrosinase isolated from *Sinapis alba* seeds (Wittstock lab) instead of crude extract. The dichloromethane extract was concentrated under an air stream and the samples were analyzed by GC-MS, using an Agilent 6890 N series gas chromatograph with an ZB-5MS column (30 m × 0.25 mm × 0.25 µm (Phenomenex, USA) and coupled with 5975 B inert XL EI / CI mass spectrometer (Agilent), splitless injection at 200°C (injection volume 1 µl), with the following temperature program: 35°C for 3 min, 12°C/min to 280°C, 30°C/min to 300°C, and 300°C hold for 3 min as described (Kuchernig et al., 2011).

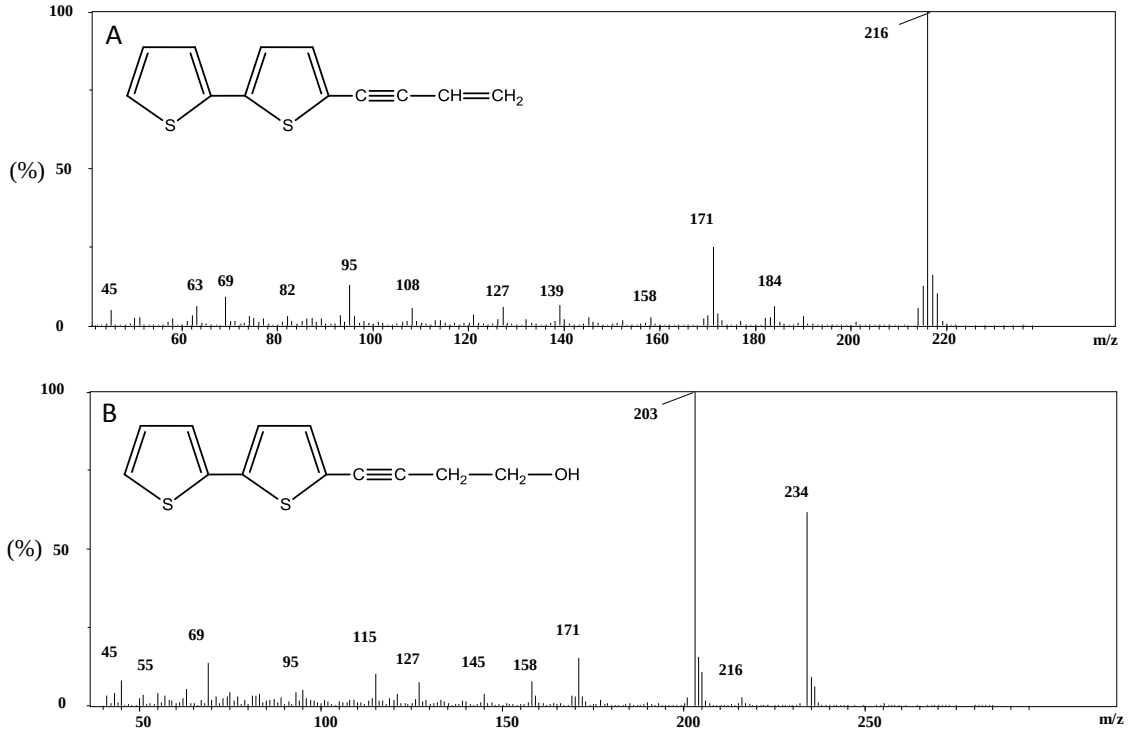
3 Results

3.1 Polyacetylene composition in tissue cultures and soil-grown plants of the Asteraceae

3.1.1 Polyacetylene composition of *Tagetes patula*

3.1.1.1 Identification and quantification of five thiophenes from *T. patula*

GC-MS methods proved to be very effective and sensitive for the separation and detection of complex mixtures of thiophenes. Fig. 3.1 shows typical mass spectra obtained upon analysis of partially purified methanolic extracts. Fragmentation patterns corresponded well with those found in the literature (Margl et al., 2002; Szarka et al., 2008) (Tab. 3.1). The analysis confirmed the presence of five thiophenes in extracts of *T. patula*. Five thiophenes were identified in partially purified methanolic extracts of soil-grown plants by GC-MS (Fig. 3.1): butenynyl-bithiophene (BBT), hydroxybutynyl-bithiophene (BBTOH), acetoxybutynyl-bithiophene (BBTOAc), pentenynyl-bithiophene (PBT), and α-terthienyl (α-T). As the thiophenes were not available as standards, quantification was based on comparison of GC-FID peak areas with that of synthetic 5-bromo-2,2'-bithiophene added as an internal standard upon extraction. FID responses were assumed to be equal for plant thiophenes and internal standard.



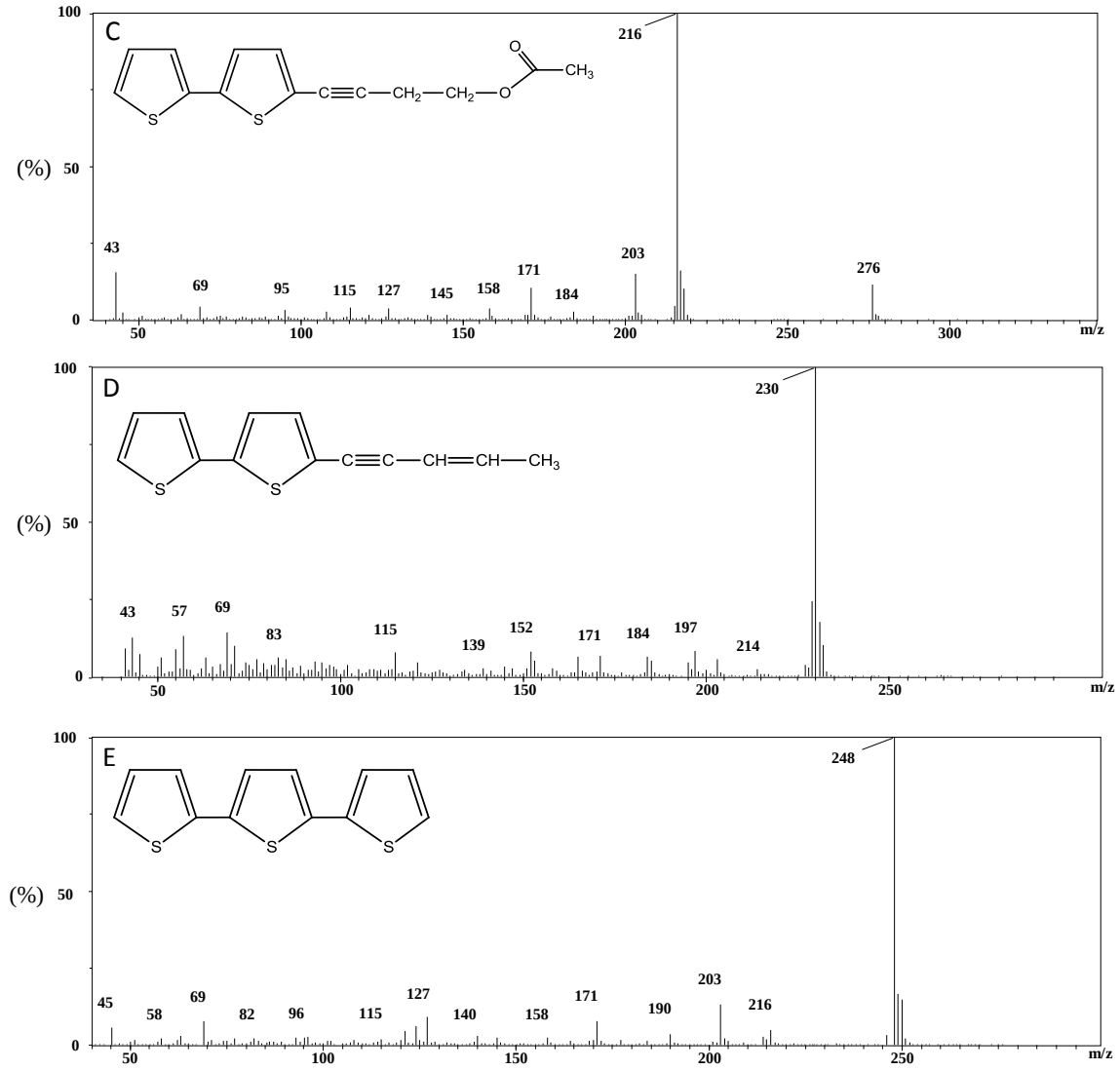


Fig. 3.1: Mass spectra of thiophenes detected by GC-MS in extracts of different organs of *T. patula* plants. A: BBT; B: BBTOH; C: BBTOAc; D: PBT; E: α-T.

Tab. 3.1: Mass spectral characteristics of thiophenes according to Margl et al (2002); Szarka et al (2006) in comparison to those obtained from extracts of soil-grown *T. patula*.

Abbreviation	Fragment ions (relative abundance (%))	
	Expected	Found
BBT	216 (100) 171 (22) 217 (16) 95 (11) 218 (10)	216 (100) 171 (25) 217 (16) 95 (13) 218 (10)
BBTOH	203 (100) 234 (76) 204 (14) 171 (21) 235 (11)	203 (100) 234 (62) 204 (15) 171 (16) 235 (9)
BBTOAc	216 (100) 43 (13) 217 (17) 203 (16) 276 (12)	216 (100) 43 (15) 217 (16) 203 (15) 276 (12)
PBT	230 (100) 229 (23) 231(18) 232 (10) 197 (9)	230 (100) 229 (24) 231 (18) 232 (10) 197 (8)
α-T	248 (100) 249 (16) 250 (14) 203 (13) 127 (14)	248 (100) 249 (16) 250 (15) 203 (13) 127 (9)

3.1.1.2 Profile of thiophenes in different organs of soil-grown *T. patula*

While all five thiophenes were detected in twelve-day-old seedlings and one-month-old plants, the composition of thiophenes differed between organs of three-month-old plants (Tab. 3.2). Leaves contained only BBT while roots and stems contained all five thiophenes. In flowers and flower buds, four and three thiophenes were found, respectively. Quantitative analysis (Fig. 3.2), showed that thiophene concentrations in seedlings and one-month-old plants are generally lower than those in three-month-old plants with the exception of leaves. There was no major difference between thiophene profiles and concentrations between seedlings and one-month-old plants if plants were extracted in total (about 6 µmol/g DW). However, thiophene concentrations were higher in below-ground organs than in above-ground organs of one-month-old plants. BBT was the most abundant thiophene in seedlings and below-ground organs of one-month-old plants with 40 % and 52 %, respectively, of the total thiophene content. PBT was the major thiophene of the above-ground organs of one-month-old plants in which it accounted for 34 % of the total thiophene content.

Roots of three-month-old plants contained 29 µmol/g DW thiophene on average, i.e. four- to fivefold higher thiophene concentrations than seedlings and one-month-old plants (Fig. 3.2). Almost 70 % of this total amount was made up by BBT and about 16 % by α-T. Flower buds were also rich in thiophenes with a total of 18 µmol/g DW, while the other organs contained less than10 µmol/g DW on average. The flower buds contained PBT as most abundant thiophene, which was accompanied by slightly lower amount of BBT and α-T. Interestingly, the thiophenes of stems of three-month-old plants contained mostly BBTOAc. In all samples analyzed BBTOAc concentrations were higher than those of BBTOH. This indicates that BBTOAc might be produced from BBTOH and not the other way around. Taken together, this analysis identified below-ground parts of *T. patula* as a rich source of BBT. In particular, three-month-old plants accumulated high BBT levels in the roots.

Tab. 3.2: Distribution of thiophenes in different organs of soil-grown *T. patula* at different growth stages.

Plant age	Plant organs	Thiophenes
Twelve days	Seedlings	BBT, BBTOH, BBTOAc, PBT, α-T
One month	All organs	BBT, BBTOH, BBTOAc, PBT, α-T
	Below-ground organs	BBT, BBTOH, BBTOAc, PBT, α-T
	Above-ground organs	BBT, BBTOH, BBTOAc, PBT, α-T
Three months	Roots	BBT, BBTOH, BBTOAc, PBT, α-T
	Flowers	BBT, BBTOAc, PBT, α-T
	Flower buds	BBT, PBT , α-T
	Stems	BBT, BBTOH, BBTOAc, PBT, α-T
	Leaves	BBT

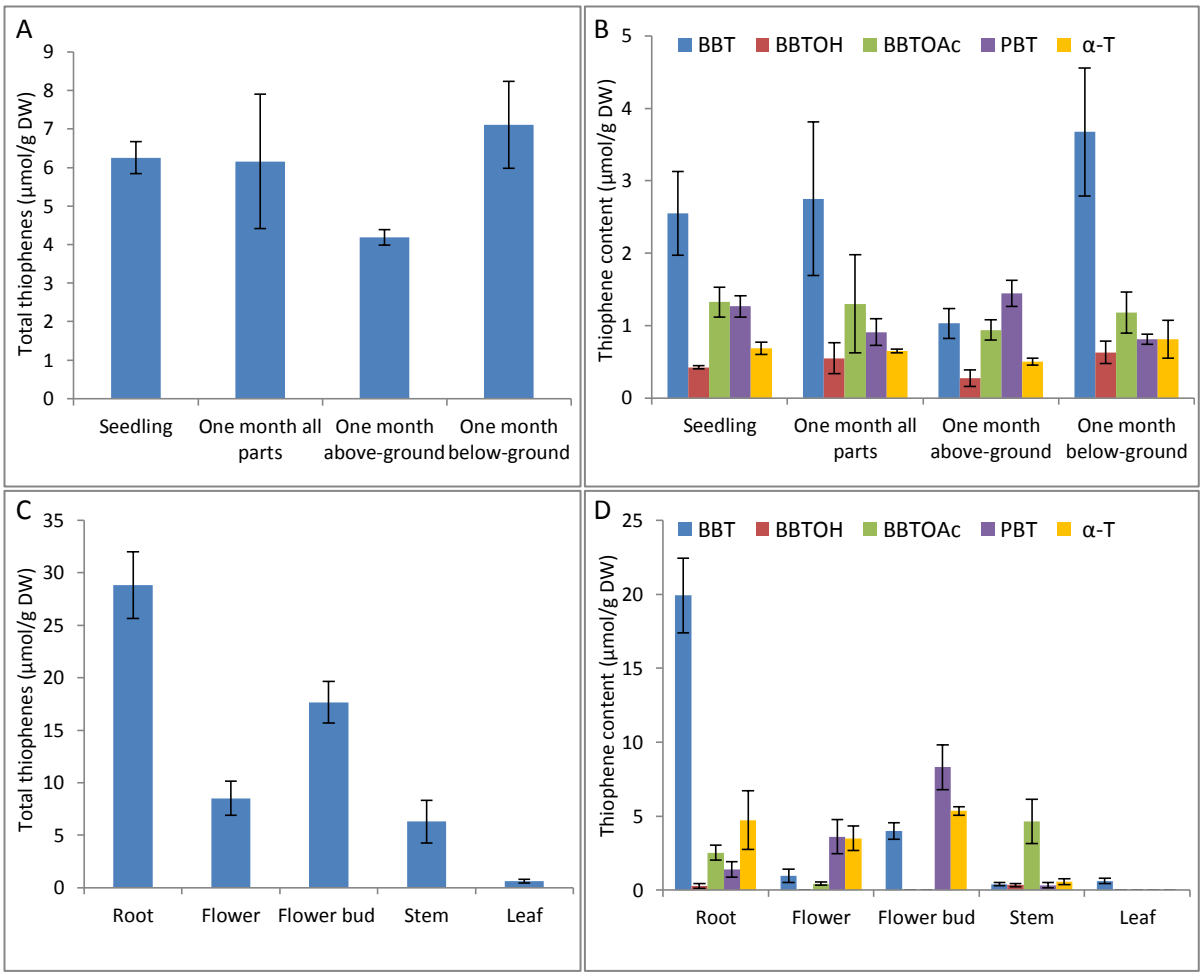


Fig. 3.2: Thiophene content in different organs of *T. patula*. Crude methanol-water extracts were partially purified by extraction with Hexane/TBME (1:1), and organic phases analyzed by GC-FID. A, C: Total amount of thiophenes; B, D: Content of individual thiophenes. C, D: Three-month-old plants. Shown are means ± SD of three independent experiments (n=3). Ab-

breviation: BBT, Butenynyl-bithiophene; BBTOH, Hydroxybutynyl-bithiophene; BBTOAc, Acetoxybutynyl-bithiophene; PBT, Pentenynyl-bithiophene; α-T, α-terthienyl.

3.1.1.3 Thiophene content in *T. patula* root cultures

The root culture of *T. patula* was established from roots of seedlings and maintained in MS medium without addition of phytohormones (Fig. 3.3 A). To identify the thiophenes, partially purified methanolic extracts of thirteen-day-old root culture were analyzed by GC-MS. Five thiophenes were identified (BBT, BBTOH, BBTOAc, PBT and α-T). This result was in agreement with (Croes et al., 1989; Menelaou et al., 1991). The same thiophenes had been detected in the soil-grown *T. patula* (3.1.1.2).

Biomass and thiophene production by the root culture were monitored for a period of 35 days. As shown in Fig. 3.3 B, the biomass increased slowly after subculturing reaching a maximum (0.60 g DW) on day 28. After day 28 biomass decreased, the roots became dark brown and less viable. As shown in Fig. 3.3 B, thiophene accumulation was decreased at the beginning maybe due to the transfer into fresh medium. From day 7 onwards thiophenes accumulation increased steadily to reach a level of almost 120 µmol/g DW on day 35 (Fig. 3.4). Major thiophenes were BBT and α-T (40 % and 41 % of total). The medium was also extracted to see whether thiophenes migrate into the medium. However, only trace amounts of α-T and BBT were detected in extracts of the culture medium. Taken together, root cultures accumulated about three to fourfold higher concentration of thiophenes than roots of three-month-old *T. patula* plants. Moreover, they grew well and the amount of biomass that can be obtained within one month was relatively high.

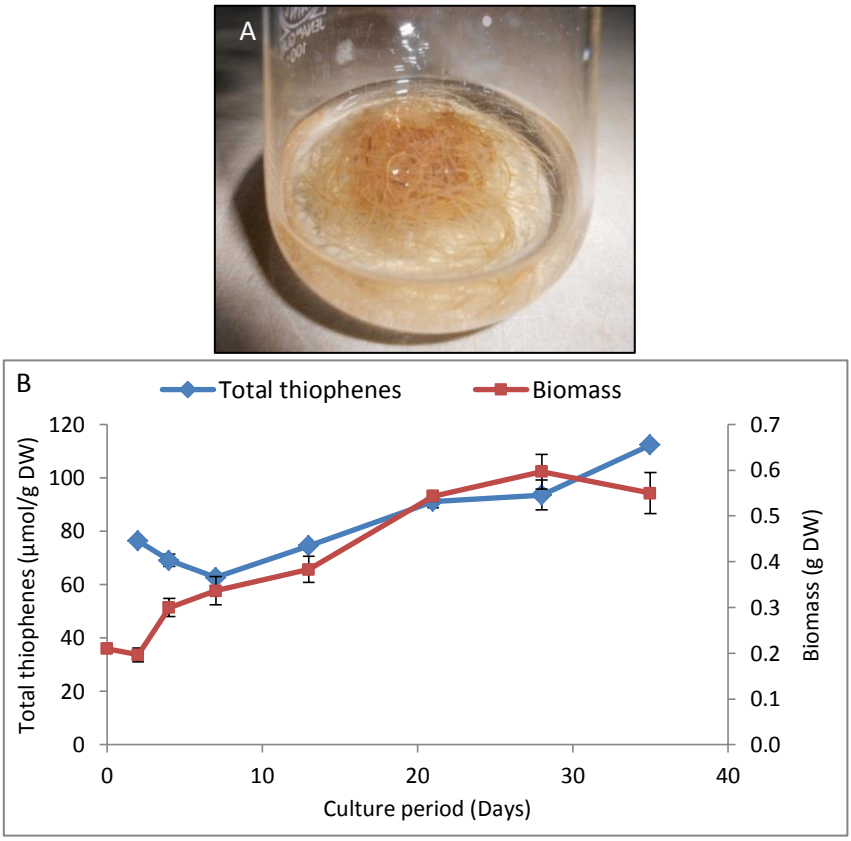


Fig. 3.3: Root culture of *T. patula*. A: Thirteen-day-old root culture; B: Growth and total thiophene accumulation in the root culture. Data shown are means ± SD of three independent experiments (n=3).

3.1.1.4 Thiophene content in *T. patula* callus culture

Callus culture was established from pieces of leaves. As shown in Fig. 3.4, callus induction was observed within a month of culture when the MS medium was supplemented with 2 mg/l 2,4-D and 2 mg/l kinetin (Rajasekaran et al., 2003), while no good callus induction was observed when the medium was supplemented with lower hormone concentration.

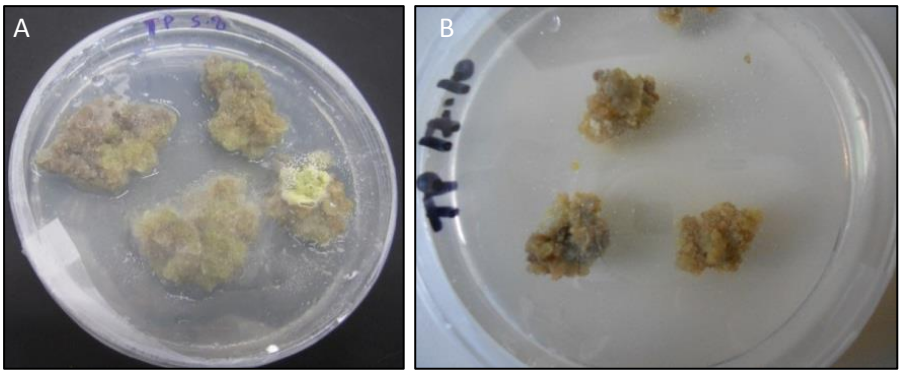


Fig. 3.4: Establishment of *T. patula* callus culture from leaf explants. A: Stabilized callus culture on solid MS medium supplemented with 2 mg/l 2,4-D and 2 mg/l kinetin; B: Non-

sufficient callus induction after 4 weeks on solid MS medium supplemented with 0.01 mg/l NAA, 0.45 mg/l kinetin, and 1 mg/l 2,4-D.

To identify thiophenes in the callus culture, thirteen-day-old callus was extracted and analyzed by GC-MS. Three thiophenes, namely BBT, PBT and α-T were detected. The hydroxylated and acetylated BBT-derivatives were not detected. Quantitative analysis showed that thiophenes accumulated in only small amounts (about 1.5 µmol/g DW) (Fig. 3.5). The major thiophene in callus was BBT which accounted for approximately 60 % of the total content. Taken together, the callus culture did not appear to be a good system for studying the biosynthesis of thiophenes.

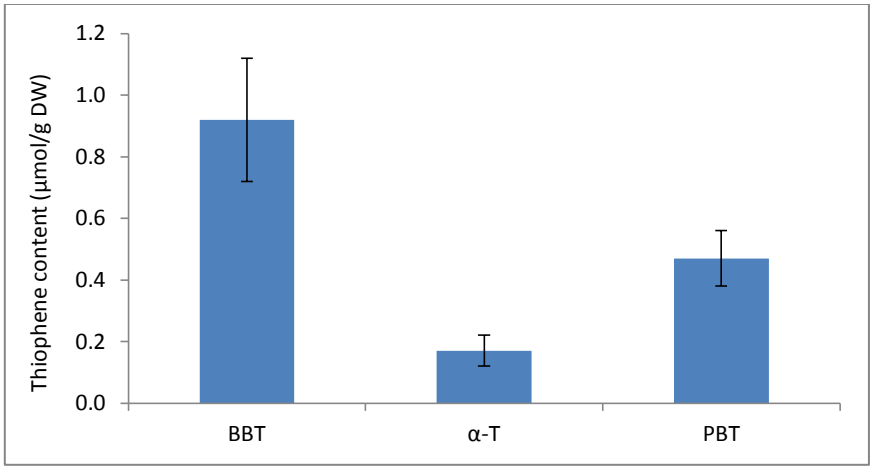


Fig. 3.5: Thiophene content in thirteen-day-old callus culture of *T. patula*. Each value is a mean ± SD of two independent experiments (n=4).

3.1.1.5 Thiophene content in *T. patula* hairy root cultures

Hairy root cultures were established by transformation of leaves of aseptically grown *T. patula* with *A.rhizogenes* LBA 1334. The inoculated leaves produced a number of hairy roots at the site of infection after about two weeks (Fig. 3.6), while no hairy root formation was observed in uninfected control leaves. Several hairy root lines were obtained. The growth rate of the lines was variable, but generally high. Based on the biomass production, two hairy root lines (line 2 and line 3) were selected for further experiments. Integration of the T-DNA into the *T. patula* genome was confirmed for the two lines by PCR using *rol*B specific primers (Fig. 3.7).

Fig. 3.6: Induction of hairy roots from *T. patula* by using *A. rhizogenes* LBA 1334. A: Hairy roots arising from leaves at the site of infection; B: Hairy root line 2 growing in solid B5 medium; C: Hairy root line 3 growing in solid B5 medium.

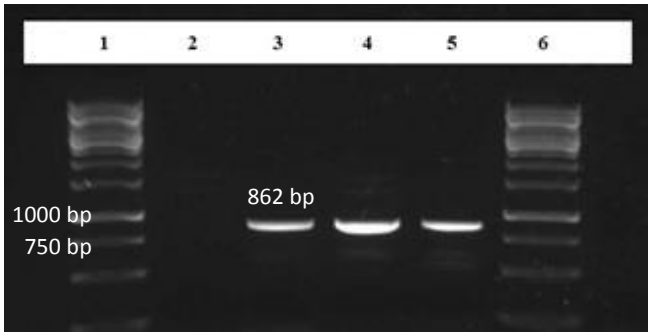


Fig. 3.7: PCR amplification of *rolB* gene (862 bp fragment) for confirmation of T-DNA transfer into hairy root lines of *T. patula*. Amplification products were separated by electrophoresis on 1 % (w/v) agarose gel stained with ethidium bromide and visualized under a UV-trans-illuminator. Lane 1, DNA marker (1 kbp); lane 2, PCR on DNA from non-transformed root (negative control); lane 3 and 4, PCR on DNA from hairy root line 2 and line 3, respectively; lane 5, PCR on plasmid DNA from *A. rhizogenes* LBA 1334 (positive control); lane 6, DNA marker (1 kbp).

To identify thiophenes in the hairy root cultures, lines 2 and 3 were extracted and the partially purified extracts were analyzed by GC-MS. Five thiophenes (BBT, BBTOH, BBTOAc, PBT, α-T) were detected in both lines. Thus, hairy roots produce the same thiophenes as non-transformed roots. When the medium of the two lines was extracted, only trace amounts of α-T were detected.

Biomass and thiophene production by lines 2 and 3 were monitored for a period of 35 and 28 days, respectively. The two lines had different growth rates. For line 2, a typical growth curve was obtained with a lag phase till day 7 after the subculturing (Fig. 3.8A). After the lag phase, cell cultures started to grow actively, entering the linear phase of growth on day 7 and reaching a maximum biomass of 0.57 g DW on day 28. After day 28, the stationary phase started and the cells became brown and lost viability. For line 3, a typical growth curve

(Fig. 3.8 B) also started with a lag phase until day 7. After the lag phase, the roots biomass increased steadily up to day 13 at which a maximum of 0.49 g DW was reached. There was no further growth after day 13. After day 21, a decrease in biomass was noticed and the cells died as indicated by the dark brown color of the culture.

As presented in Fig. 3.8, the total thiophene content increased as soon as the cultures passed the lag phase, reaching a maximum of 76 µmol/g DW and 86 µmol/g DW on day 13 for line 2 and line 3, respectively. In line 2, there was no further increase in total thiophene content and thiophene levels dropped from day 28 on. In line 3, total thiophene content started to increase again at day 21, and a level of more than 100 µmol/g DW was reached on day 28. The major thiophene in lines 2 and 3 was BBT which made up about 70 % of the total content. Taken together, the two hairy root lines grew well and accumulated similar levels of thiophene as root cultures. As it was easier to maintain these cultures over longer periods than normal root cultures, hairy root cultures were used for elicitation experiments.

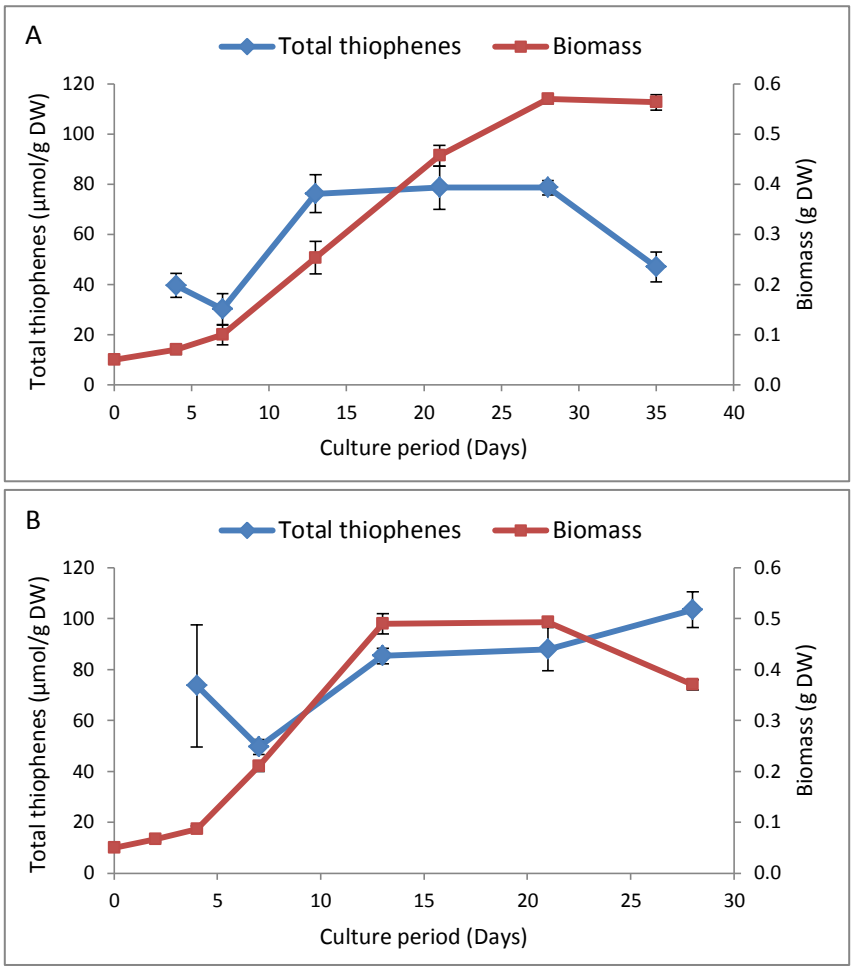


Fig. 3.8: Growth and total thiophene content in two hairy root lines of *T. patula*. A: Line 2; B: Line 3. Values are means ± SD of three independent experiments (n=3).

3.1.1.6 Thiophene profiles of *T. patula* hairy root cultures upon elicitation

To identify best elicitation conditions, different concentrations of elicitors were tested and the time point and duration of induction was varied. As a first step, the effect of elicitors on thiophene accumulation of hairy root lines 2 and 3 (thirteen-day-old) were investigated after addition of different concentrations of MeJ or chitosan for 72 h or vanadyl sulfate for 48 h (Fig. 3.9 and Fig. 3.10). In line 2, the highest thiophene levels were reached with 100 µM MeJ corresponding to a 1.8-fold induction of total thiophenes as compared to the control. However, a loss of biomass was noticed. When individual thiophenes were analyzed, highest induction (3.7-fold) was seen for α-T and moderate (1.8-fold) induction for BBT. Chitosan (25-100 mg/l) and vanadyl sulfate (1-50 mg/l) did not have major effects on thiophene accumulation in line 2. In line 3, vanadyl sulfate at 30 mg/l was the best inducer of total thiophenes. It increased total thiophene levels 1.51-fold as compared to control cultures. The increase was due to an increase of only BBT and α-T (1.6 and 1.5-fold induction, respectively). No difference in biomass was observed between the control roots and the roots elicited with 30 mg/l vanadyl sulfate. Based on these results, 100 µM MeJ and 30 mg/l vanadyl sulfate were selected as an optimum concentration for elicitation of total thiophene content in line 2 and line 3, respectively, and used in all subsequent experiments.

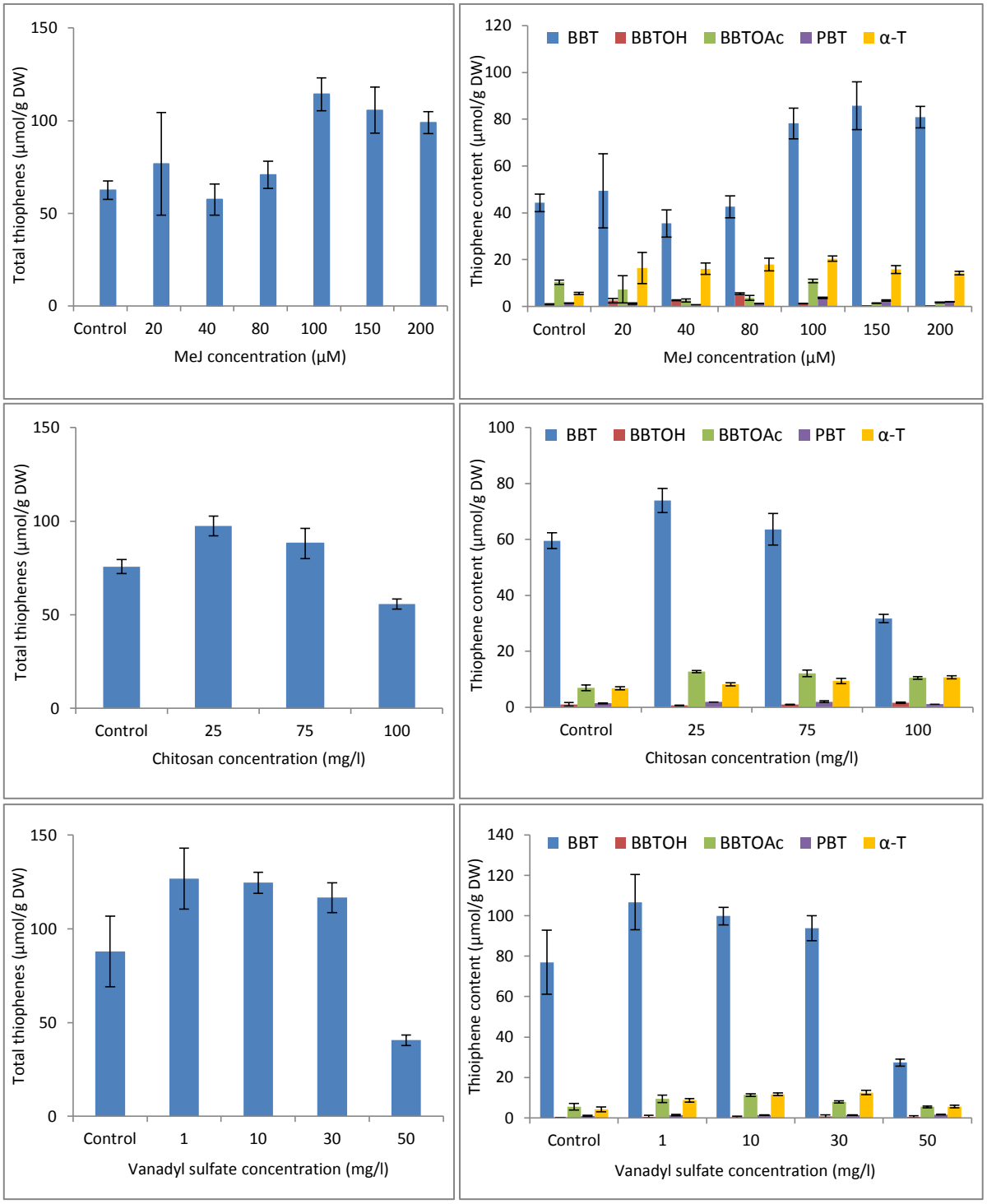


Fig. 3.9: The content of thiophenes in thirteen-day-old line 2 hairy root cultures of *T. patula* when challenged with different types and concentrations of elicitors. Values are means ± SD of three independent experiments (n=3).

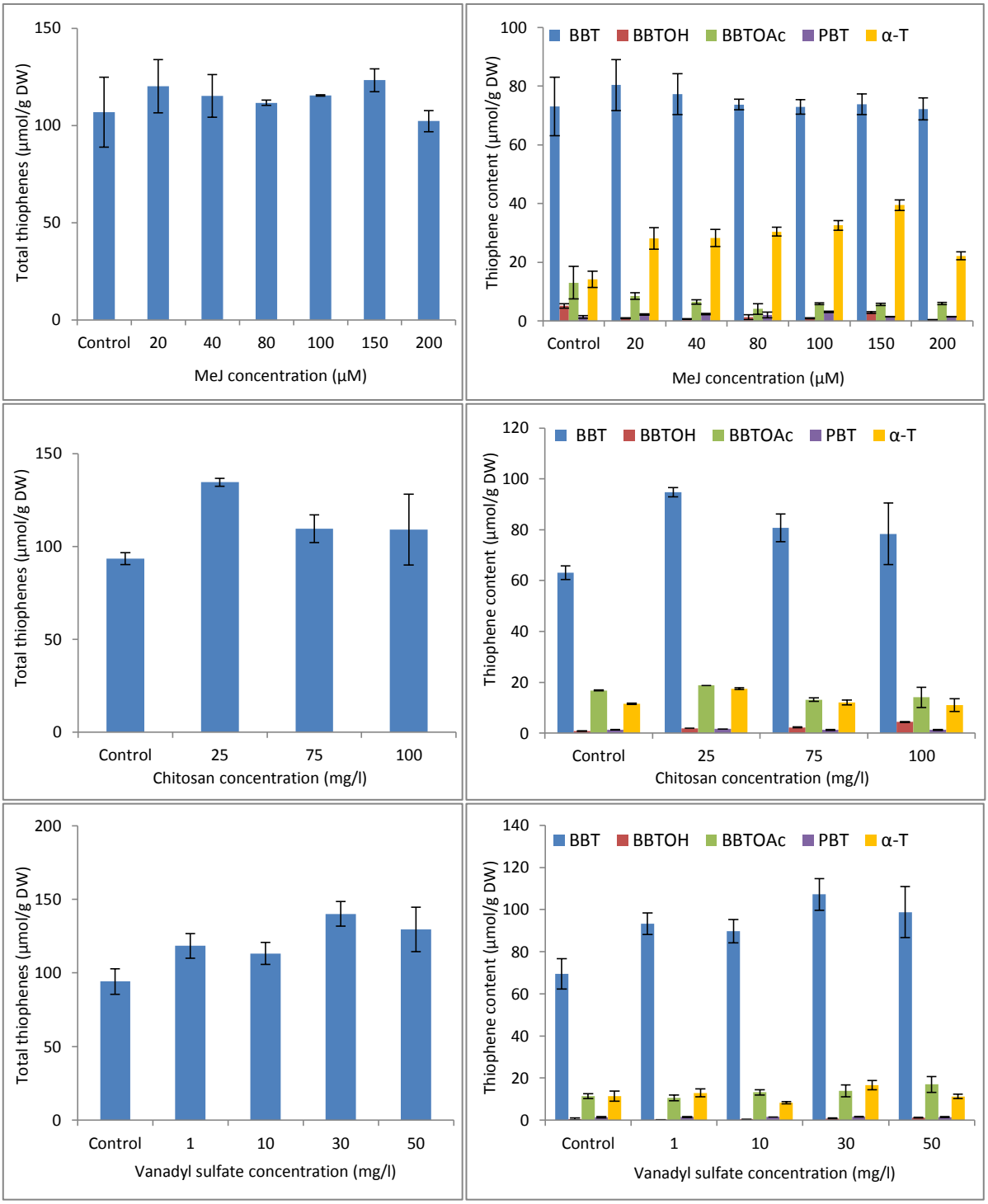


Fig. 3.10: The content of thiophenes in thirteen-day-old line 3 hairy root cultures of *T. patula* when challenged with different types and concentrations of elicitors. Values are means ± SD of three independent experiments (n=3).

Next, the effect of the culture age at time of induction was studied for lines 2 and 3. For line 2, 100 µM MeJ were added at 7, 13, 21 and 28 days after subculture, i.e. at the early, middle and late growth phase. Hairy roots were harvested 72 h after elicitor addition. The most responsive stage for thiophene production was day 21 with an about 1.78-fold increase

of total thiophene content compared to the untreated cultures (Fig. 3.11A). For line 3, 30 mg/l vanadyl sulfate was added to the cultures 4, 7, 13 and 21 days after subculture representing the early, middle, late growth phase and the end of the stationary phase. Hairy roots were harvested 48 h after elicitor addition. The most responsive stage for thiophene production was day 7 with an about 1.5-fold increase in total thiophene content compared to the untreated cultures (Fig. 3.11B).

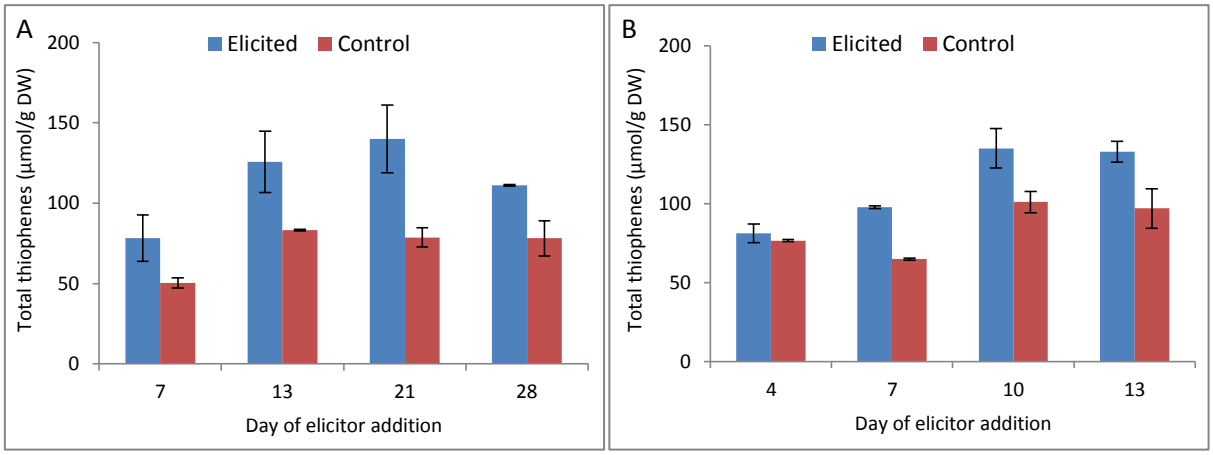


Fig. 3.11: The content of thiophenes in hairy root lines of *T. patula* after addition of elicitor at different days after subculture. A: Line 2 exposed to 100 µM MeJ for 72 h; B: Line 3 exposed to 30 mg/l vanadyl sulfate for 48 h. Shown are means ± SD of three independent experiments (n=3).

After the optimal type, concentration and time point of elicitor addition had been selected, the effect of contact time of elicitor with the culture was investigated. Hairy roots were harvested 24, 48, or 72 h after addition of 100 µM MeJ on the day 21 (line 2) or 30 mg/l vanadyl sulfate on the day 7 (line 3) after subculture. For both lines, the maximum elicitation was observed after the hairy roots had been exposed for 72 h to the elicitor (Fig. 3.12). Taken together, an twofold increase of total thiophene content was achieved in line 2 by treatment with 100 µM MeJ for 72 h starting on day 21 after subculture. In line 3, an almost twofold increase was achieved by treatment with 30 mg/l vanadyl sulfate on day 7 after subculture.

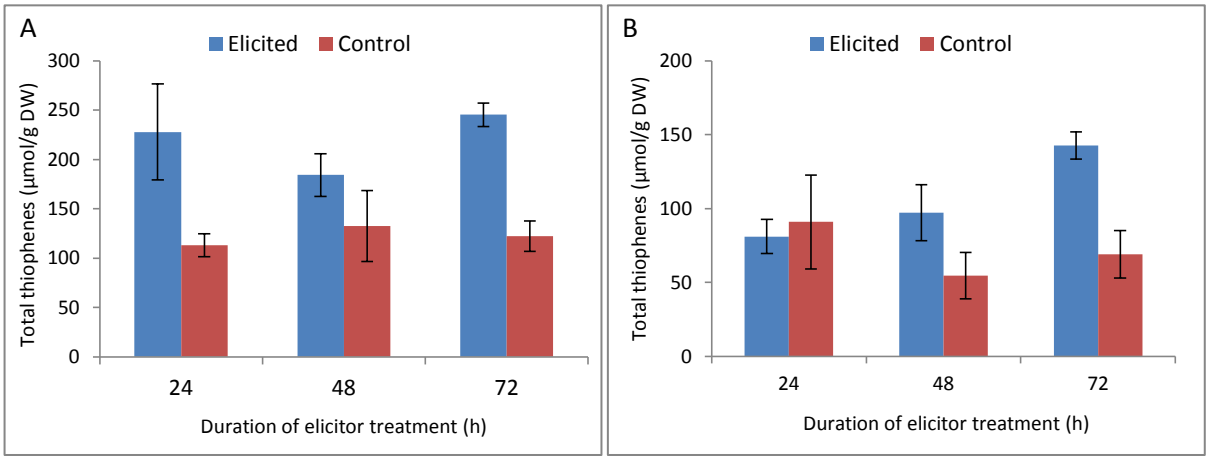


Fig. 3.12: The content of thiophenes in hairy root lines of *T. patula* when challenged with elicitor for different time periods. A: Line 2 elicited with 100 µM MeJ on day 21 after subculture; B: Line 3 elicited with 30 mg/l vanadyl sulfate on day 7 after subculture. Shown are means ± SD of three independent experiments (n=3).

3.1.2 Polyacetylene composition of *Arctium lappa*

3.1.2.1 Identification and quantification of polyacetylenes from *A. lappa*

GC-MS analysis was conducted to identified 1-tridecene-3,5,7,9,11-pentayne (T1e-3,5,7,9,11y) and 1,11-tridecadiene-3,5,7,9-tetrayne (T1,11e-3,5,7,9y), known to occur in *A. lappa* (Schulte et al., 1967; Takasugi et al., 1987), in extracts of different organs of *A. lappa*. Identification was based on mass spectra as compared to those published previously (Takasugi et al., 1987; Binder et al., 1990a). Fig. 3.13 and Tab. 3.3 show the mass fragmentation of compounds detected by GC-MS in comparison with the mass fragmentation of polyacetylenes from the literature. As all fragments reported previously were detected and occurred with similar abundances as reported (Tab. 3.3), this confirmed the presence of T1e-3,5,7,9,11y and T1,11e-3,5,7,9y in extracts of *A. lappa*. As the two polyacetylenes were not available as standards, quantification was based on comparison of GC-FID peak areas with that of synthetic 4-chlorobenzophenone added as an internal standard upon extraction. FID responses were assumed to be equal for plant polyacetylenes and internal standard. Other internal standards were tested but proved to be unsuitable due to co-elution with compounds of interest (5-bromo-2,2'-bithiophene), very different retention time (2-bromothiophene) or inability to detect the compound after extraction with the GC-method (1-tridecene).

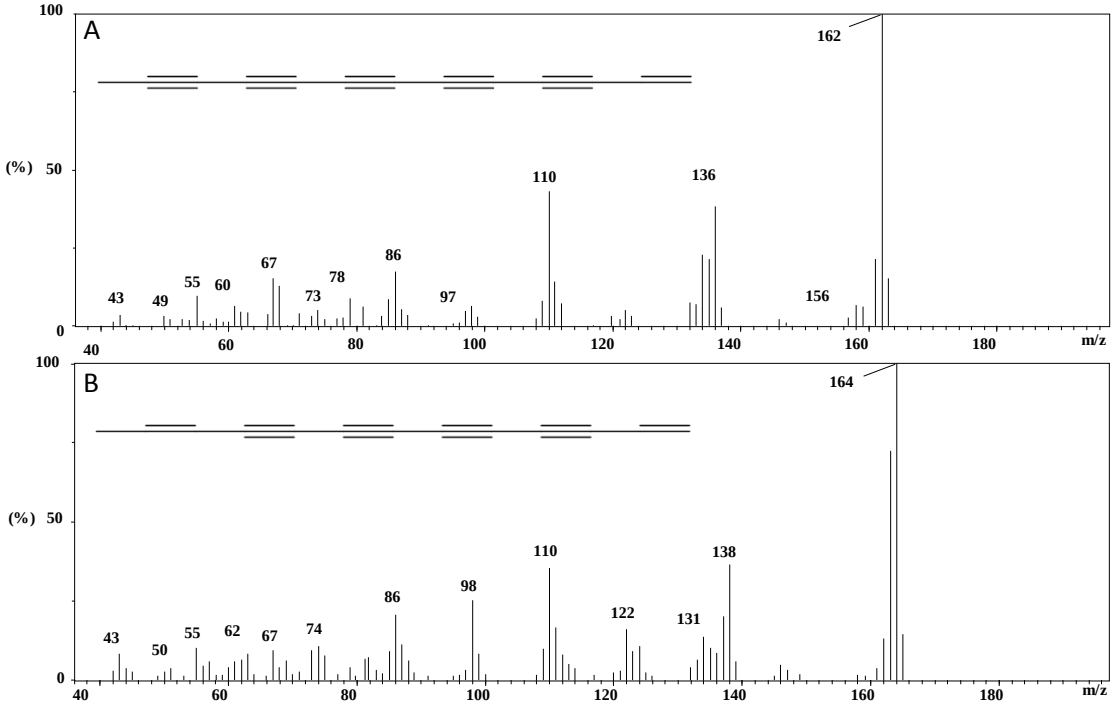


Fig. 3.13: Mass spectra of polyacetylenes detected by GC-MS in extracts of different organs of soil-grown *A. lappa* plants. A: T1e-3,5,7,9,11y; B: T1,11e-3,5,7,9y.

Tab. 3.3: Mass spectral characteristics of polyacetylenes according to Takasugi et al (1987); Binder et al (1990a) in comparison to those obtained from extracts of soil-grown *A. lappa*.

Abbreviation	Fragment ions (relative abundance (%))	
	Expected	Found
T1e-3,5,7,9,11y	163 (14) 162 (100) 161 (20) 136 (38) 135 (16) 134 (18) 110 (42) 98 (2) 86 (13)	163 (14) 162 (100) 161 (21) 136 (38) 135 (21) 134 (23) 110 (43) 97 (6) 86 (17)
T1,11e-3,5,7,9y	165 (18) 164 (100) 163 (69) 139 (3) 138 (38) 137 (11) 110 (30) 98 (17) 86 (11)	165 (14) 164 (100) 163 (72) 139 (6) 138 (36) 137 (20) 110 (35) 98 (25) 86 (20)

3.1.2.2 Profile of polyacetylenes in different organs of soil-grown *A. lappa*

Polyacetylene composition was studied in fifteen-day-old seedlings, one-month-old, and three-month-old plants (Tab. 3.4). No polyacetylene was detected in seedlings. Both T1e-3,5,7,9,11y and T1,11e-3,5,7,9y were present in below-ground parts of one-month-old plants while above-ground parts contained only T1,11e-3,5,7,9y. Of three-month-old plants, secondary roots contained polyacetylenes (T1e-3,5,7,9,11y and T1,11e-3,5,7,9y) while no polyacetylenes were detectable in primary roots and leaves. Quantitative analysis (Fig. 3.14), showed that polyacetylene levels in all analyzed samples were very low (< 1 µmol/g DW). In one-month-old plants, levels in below-ground organs were higher than those in above-ground organs. Highest levels were found in secondary roots of three-month-old plants. The two polya-

cetylenes each accounted for about 50 % of the total content. Taken together, *A. lappa* accumulate only very low levels of T1e-3,5,7,9,11y and T1,11e-3,5,7,9y in the growth stages analyzed. Polyacetylene levels depended on growth stage and organ.

Tab. 3.4: Distribution of polyacetylenes in different organs of soil-grown *A. lappa* at different growth stages.

Plant old	Plant organs	Polyacetylenes	
		T1e-3,5, 7,9,11y	T1,11e-3,5,7,9y
Fifteen days	Seedlings		
One month	All organs	×	×
	Above ground organs		×
	Below ground organs	×	×
Three months	Primary roots		
	Secondary roots	×	×
	Leaves		

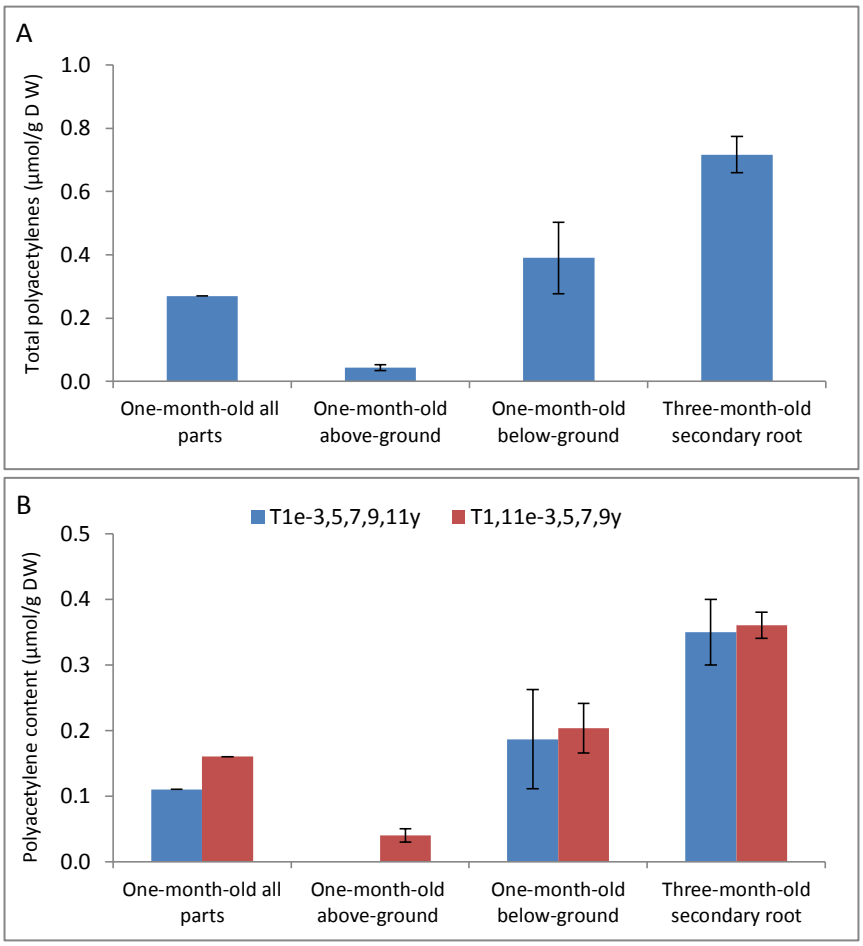


Fig. 3.14: Polyacetylene content in different organs of *A. lappa*. Crude methanol-water extracts were partially purified by extraction with Hexane/TBME (1:1), and organic phases analyzed by GC-FID. A: Total amount of polyacetylenes; B: Content of individual polyacetylenes. Shown are means ± SD of three independent experiments (n=3).

3.1.2.3 Polyacetylene content in *A. lappa* root cultures

The root culture of *A. lappa* was obtained from roots and maintained in MS medium containing 0.2 mg/l NAA. Without addition of NAA, callus-like structures were formed at the tip of the roots (Fig. 3.15A). When the roots were maintained in MS medium supplemented with 1 mg/l NAA and 1 mg/l IBA or 1 mg/l NAA, growth was good and fast at the beginning but after two weeks callus formation began. After three weeks all the roots were converted to callus (Fig. 3.15 B).

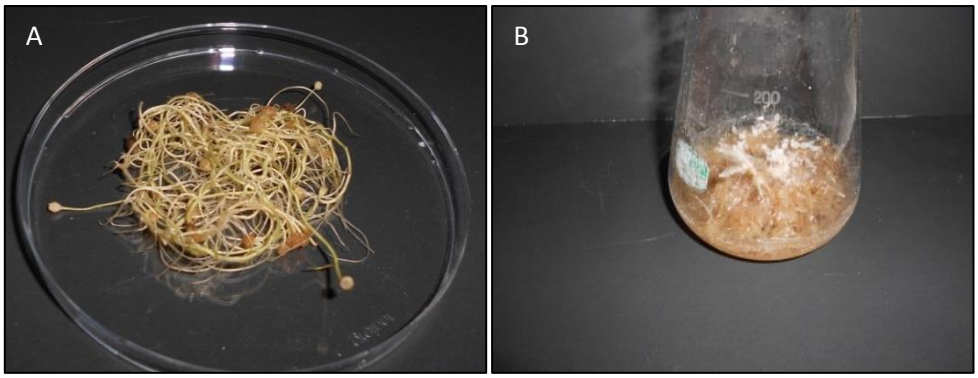


Fig. 3.15: Root culture of *A. lappa*. A: Callus-like structures at the tips of roots maintained in MS medium; B: Callus observed when the roots were maintained in MS medium supplemented with 1 mg/l NAA and 1 mg/l IBA.

To identify the polyacetylenes in the root culture, partially purified methanolic extracts of thirteen-day-old root cultures were analyzed by GC-MS. Two polyacetylenes were identified in the root culture: T1e-3,5,7,9,11y and T1,11e-3,5,7,9y. These two polyacetylenes are the same as those found in soil-grown plants (3.1.2.2). Quantitative analysis of the extracts by GC-FID showed that the total amount of the two polyacetylenes was very low (0.27 µmol/ g DW) (Fig. 3.16). The two polyacetylenes accumulated to about the same extent.

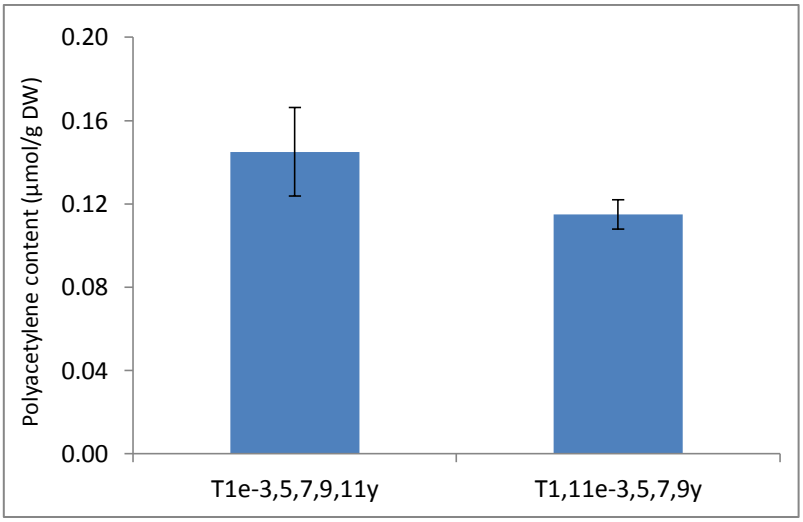


Fig. 3.16: Polyacetylene content in thirteen-day-old root cultures of *A. lappa*. Values are means ± SD of three independent experiments (n=3).

The root culture was studied in terms of biomass production for a period of 35 days. As shown in Fig. 3.17, the biomass of roots increased slowly after subculturing and a maximum biomass of 0.31 g DW was reached on day 21 after subculture. Because of this low biomass production, only few further experiments were conducted with the root cultures.

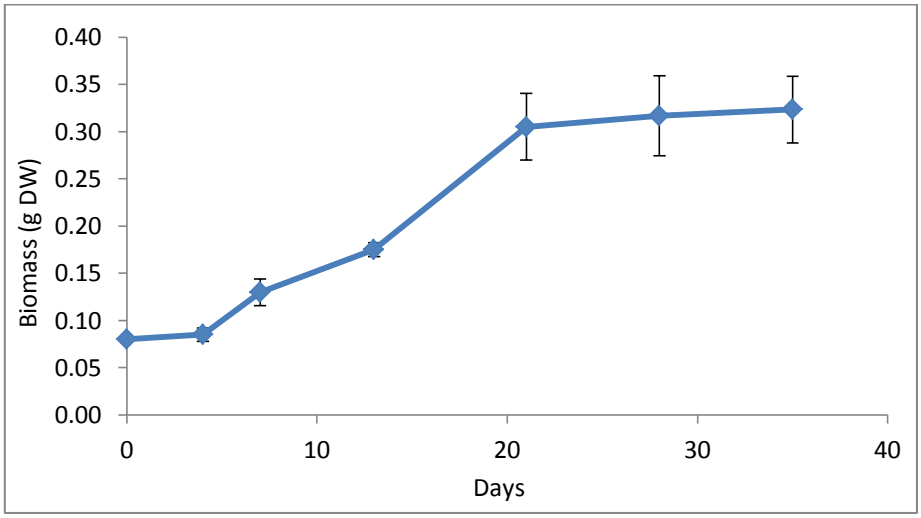


Fig. 3.17: Growth of *A. lappa* root cultures. Values are means ± SD of three independent experiments (n=3).

In order to test if polyacetylene production can be induced chemically, 100 mg/l chitosan and 100 µM MeJ were added individually to fifteen-day-old cultures (Fig. 3.18). Cultures were exposed to the elicitor for 72 h. Addition of chitosan led to a 2.2-fold increase of polyacetylene content to about 1 µmol/g DW. MeJ did not affect polyacetylene content. Taken together, root cultures of *A. lappa* accumulated only very small levels of polyacetylenes (< 1 µmol/g DW). It was possible to induce polyacetylene production twofold in fifteen-day-old

cultures by supplementation with 100 mg/l chitosan for 72 h. As the cultures also did not produce enough biomass, they are however, not suitable for studies on polyacetylene biosynthesis.

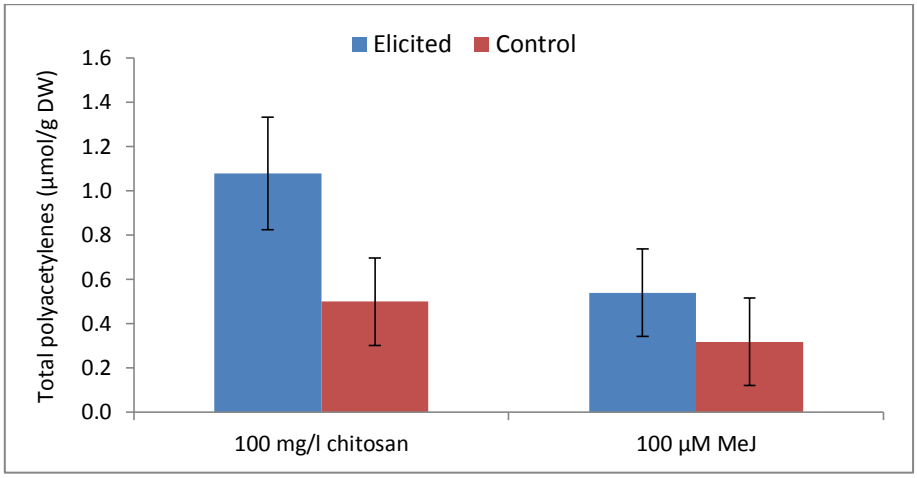


Fig. 3.18: Content of polyaceytylenes upon addition of elicitors to fifteen-day-old root cultures of *A. lappa*. Values are means ± SD of two independent experiments (n=2).

3.1.2.4 Polyacetylenes in *A. lappa* callus culture

The callus culture of *A. lappa* originated from leaves maintained on solid MS medium supplemented with 2 mg/l 2,4-D and 2 mg/l BA (He et al., 2006). The callus appeared after two weeks (Fig. 3.19). When the leaves were maintained on solid MS medium supplemented with 2 mg/l 2,4-D and 2 mg/l kinetin, explants did not show any sign of callus formation within four weeks. For identification of polyacetylenes in the callus culture, thirteen-day-old cultures were extracted and analyzed by GC-MS. No polyacetylene was detected. Thus, the callus culture of *A. lappa* does not appear to be a good system to study the biosynthesis of polyacetylenes.

Fig. 3.19: Callus culture of *A. lappa* on solid MS medium supplemented with 2 mg/l 2,4-D and 2 mg/l BA.

3.1.2.5 Polyacetylenes in *A. lappa* callus suspension culture

Callus suspension cultures were established and maintained in MS medium supplemented with 2 mg/l 2,4 D and 2 mg /l BA. Cells of seven-day-old cultures were harvested and their partially purified methanolic extracts were analyzed by GC-MS. No polyacetylene was detected. The medium was also extracted and analyzed, but no polyacetylene was detected. Before further experiments on elicitation were conducted, growth of the culture over time was studied. As shown in Fig. 3.20, a typical growth curve was obtained with a lag phase until day 13. After the lag phase, cell cultures grew faster and reached a maximum biomass (0.92 g DW) on day 21. After that, biomass did not increase any further. Between day 28 and day 35 biomass decreased and the cells became dark brown.

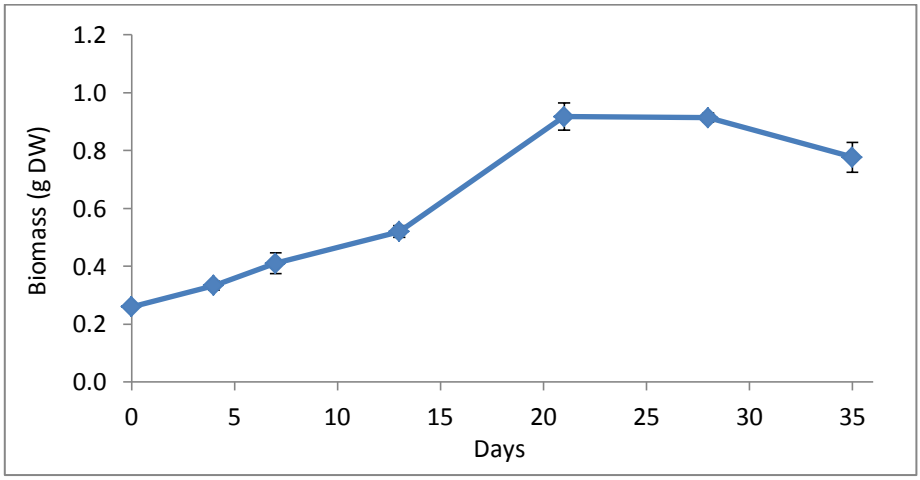


Fig. 3.20: Growth of *A. lappa* callus suspension culture. Values are the means ± SD of two independent experiments (n=4).

For inducing the synthesis of polyacetylenes in the callus suspension culture, different concentrations of MeJ (40-400 µM), chitosan (25-300 mg/l), vanadyl sulfate (30-200 mg/l) and copper sulfate (1-100 mg/l) were individually added to sixteen-day-old cultures. The cultures were exposed to MeJ and chitosan for 72 h, to vanadyl sulfate and copper sulfate for 48 h. Despite addition of elicitors, no polyacetylenes were detectable.

3.1.2.6 Polyacetylenes in *A. lappa* hairy root cultures

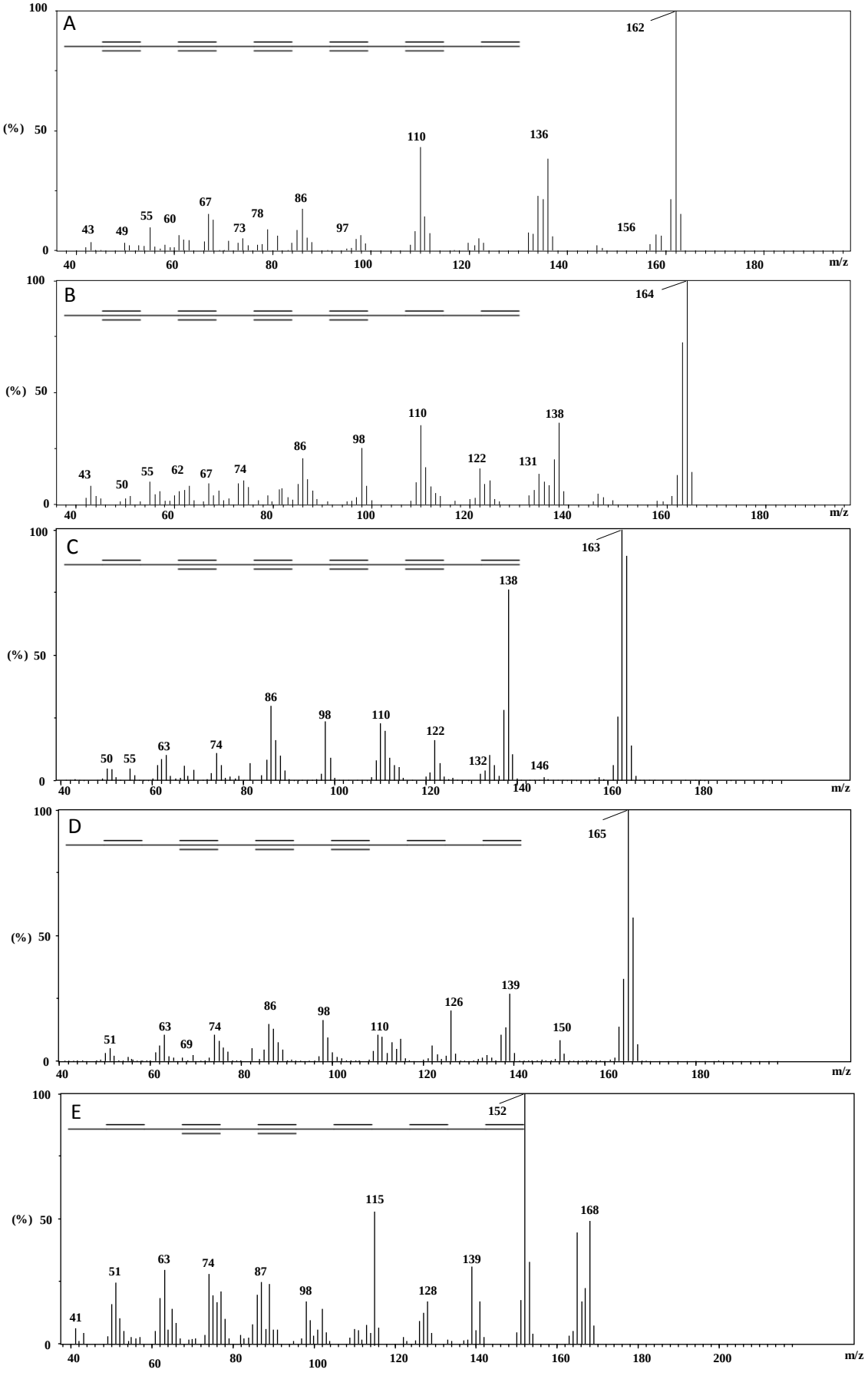
Leaf explants of aseptically grown plants were used to induce hairy roots by transformation with *A. rhizogenes* LBA 1334 and *A. rhizogenes* DSMZ 3020. Within two weeks, the leaf explants turned brown, died and no hairy root formation was observed (Fig. 3.21).

Fig. 3.21: Leaf explants of aseptically grown *A. lappa* two weeks after inoculation with *A. rhizogenes* LBA 1334.

3.1.3 Polyacetylene composition of *Carthamus tinctorius*

3.1.3.1 Identification and quantification of polyacetylenes from *C. tinctorius*

GC-MS analysis was conducted to identify polyacetylenes in partially purified methanolic extracts of soil-grown plants. Identification was based on mass spectra as compared to those published previously (Binder et al., 1990a). Five polyacetylenes were identified: 1-tridecene-3,5,7,9,11-pentayne (T1e-3,5,7,9,11y), 1,3-tridecadiene-5,7,9,11-tetrayne (T1,3e-5,7,9,11y), 1,11-tridecadiene-3,5,7,9-tetrayne (T1,11e-3,5,7,9y), 1,3,11-tridecatriene-5,7,9-triyne (T1,3, 11e-5,7,9y), and 1,3,5,11-tridecatetraene-7,9-diyne (T1,3,5,11e-7,9y). Fig. 3.22 and Tab. 3.5, show the mass fragmentation of compounds detected by GC-MS in comparison with the mass fragmentation of polyacetylenes from the literature. As all fragments reported previously were detected and occurred with similar abundances as reported (Tab. 3.5), this confirmed the presence of T1e-3,5,7,9,11y, T1,3e-5,7,9,11y, T1,11e-3,5,7,9y, T1,3,11e-5,7,9y, and T1,3,5,11e-7,9y in extracts of *C. tinctorius*. As those polyacetylenes were not available as standards, quantification was based on comparison of GC-FID peak areas with that of synthetic 4-chlorobenzophenone added as an internal standard upon extraction. FID responses were assumed to be equal for plant polyacetylenes and internal standard.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110
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51
69
E
152
115
168
63
74
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87
139
98
128
41
200

Fig. 3.22: Mass spectra of polyacetylenes detected by GC-MS in extracts of different organs of *C. tinctorius* plants. A: T1e-3,5,7,9,11y; B: T1,3e-5,7,9,11y; C: T1,11e-3,5,7,9y; D: T1,3,11e-5,7,9y; E: T1,3,5,11e-7,9y.

Tab. 3.5: Mass spectral characteristics of polyacetylenes according to Binder et al., 1990a in comparison to those obtained from extracts of soil-grown *C. tinctorius*.

Abbreviation	Fragment ions (relative abundance (%))	
	Expected	Found
T1e-3,5,7,9,11y	163 (14) 162 (100) 161 (20) 136 (38) 135 (16) 134 (18) 110 (42) 98 (2) 86 (13)	163 (14) 162 (100) 161 (21) 136 (38) 135 (21) 134 (23) 110 (43) 97.90 (6) 86 (17)
T1,3e-5,7,9,11y	165 (12) 164 (90) 163 (100) 139 (9) 138 (78) 137 (24) 110 (18) 98 (18) 86 (20)	165 (13) 164 (90) 163 (100) 139 (10) 138 (76) 137 (28) 110 (22) 98 (22) 86 (30)
T1,11e-3,5,7,9y	165 (18) 164 (100) 163 (69) 139 (3) 138 (38) 137 (11) 110 (30) 98 (17) 86 (11)	165 (14) 164 (100) 163 (72) 139 (6) 138 (36) 137 (20) 110 (35) 98 (25) 86 (20)
T1,3,11e-5,7,9y	166 (60) 165 (100) 164 (29) 163 (17) 139 (29) 138 (8) 126 (11) 115 (11) 110 (7)	166 (58) 165 (100) 164 (32) 163 (13) 139 (26) 138 (14) 126 (20) 115 (9) 110 (10)
T1,3,5,11e-7,9y	168 (58) 167 (15) 166 (15) 153 (28) 152 (100) 141 (14) 139 (19) 128 (14) 115 (35)	168 (58) 167 (22) 166 (17) 153 (40) 152 (100) 141 (15) 139 (22) 128 (17) 115 (38)

3.1.3.2 Profile of polyacetylenes in different organs of soil-grown *C. tinctorius*

Polyacetylene composition was studied in fifteen-day-old seedlings, one-month-old and three-month-old plants (Tab. 3.6). In above and below-ground organs of one-month-old plants four polyacetylenes were detected. All five polyacetylenes were detected in the seedlings and in flowers of three-month-old plants. The flower buds and secondary roots of three-month old plants contained four and three polyacetylenes, respectively, while primary roots of three-month-old plants contained only a single polyacetylene (T1,11e-3,5,7,9y). No polyacetylene was detected in the stem.

Quantitative analysis (Fig. 3.23) showed that seedlings and flowers of three-month-old plants accumulate highest levels of polyacetylenes (5 µmol/g DW and 9 µmol/g DW, respectively). All other samples analyzed contained less than 2 µmol/g DW. In seedlings, T1,3,11e-5,7,9y was the most abundant polyacetylene. It accounted for approximately 50 % of total polyacetylene content while T1,3,5,11e-7,9y accounted for approximately 33 % of total content. The major polyacetylenes in flowers of three-month-old plants were T1,3e-5,7,9,11y and T1,3,11e-5,7,9y each accounted for approximately 27 % of the total content. Taken together, the analysis identified seedlings and flowers of *C. tinctorius* as an accumulation site of C_{13}-

polyacetylenes. While seedlings accumulated mostly two compounds with three and two acetylenic bonds, respectively, flowers accumulated a mixture of penta-, tetra-, and triynes.

Tab. 3.6: Distribution of polyacetylenes in different organs of soil-grown *C. tinctorius* at different growth stages.

Plant age	Plant organs	Polyacetylenes				
		T1e-3,5,7,9,11y	T1,3e-5,7,9,11y	T1,11e-3,5,7,9y	T1,3,11e-5,7,9y	T1,3,5,11e-7,9y
Fifteen days	Seedlings	×	×	×	×	×
One month	All organs	×		×	×	×
	Above ground organs	×		×	×	×
	Below ground organs	×		×	×	×
Three months	Secondary roots	×		×	×	
	Primary roots			×		
	Flowers	×	×	×	×	×
	Flower buds	×	×		×	×
	Leaves			×	×	×
	Stems					

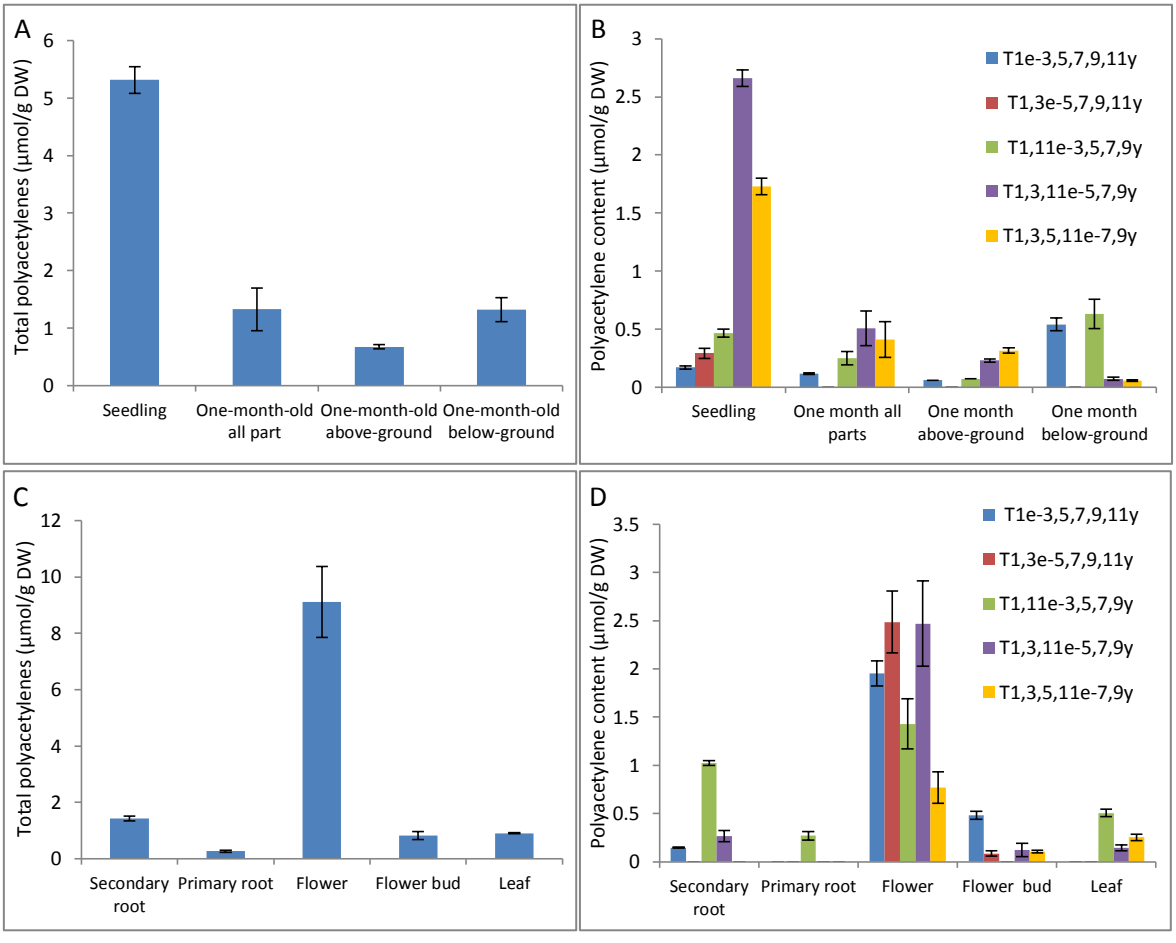

Fig. 3.23: Polyacetylene content in different organs of *C. tinctorius*. Crude methanol-water extracts were partially purified by extraction with Hexane/TBME (1:1), and organic phases analyzed by GC-FID. A, C: Total amount of polyacetylenes; B, D: Content of individual polyacetylenes. Shown are means ± SD of three independent experiments (n=3).

3.1.3.3 Polyacetylenes in *C. tinctorius* root cultures

Establishment of root culture was tried under different conditions. Roots of aseptically grown plants were excised and maintained in MS medium without addition of phytohormones. Roots grew well, but after two subcultures, roots turned black, stopped growing and died (Fig. 3.24 A-B). When the roots were maintained in MS medium supplemented with 1 mg/l NAA alone or in combination with 1 mg/l IBA and also when supplemented with 0.2 mg/l NAA, callus formation was observed after two weeks and within four weeks adventitious shoots appeared (Fig. 3.24 C). In another trial, roots were maintained in static liquid medium (Bernard et al., 2011). A small amount of MS medium (6 ml) was used in 250 ml flasks. Through four weeks no root growth was observed (Fig. 3.24 D). Other conditions were tested by increasing the amount of sucrose in MS medium (5 and 8 % sucrose) and use of MS medium without the addition of magnesium sulfate (Keil et al., 2000). In high sucrose MS medium adventitious roots formed within 3 weeks. Lack of magnesium sulfate did not promote growth within four weeks. Thus, under none of the tested conditions, root cultures of *C. tinctorius* were obtained.

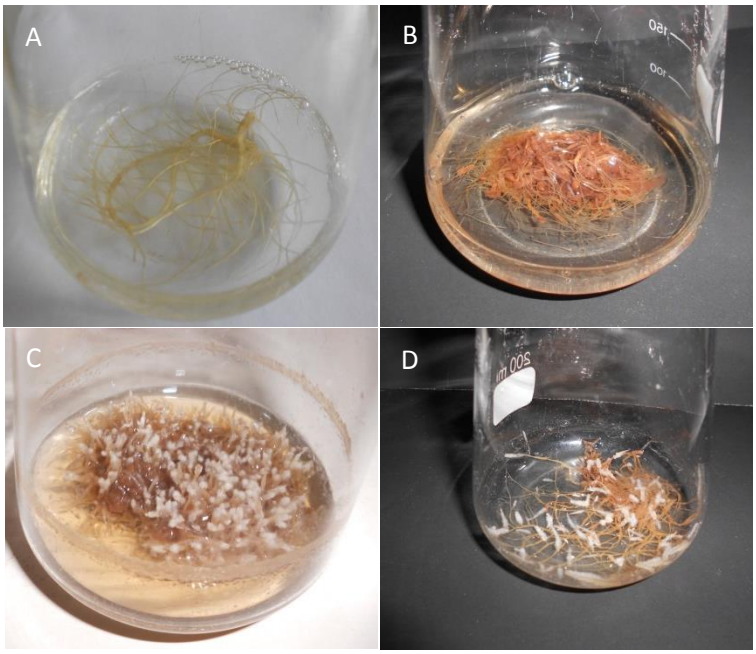

Fig. 3.24: Root cultures of *C. tinctorius*. A: Roots were excised from *in vitro* plants and maintained in MS medium without addition of phytohormones; B: Roots from A after two times subculturing; C: Adventitious root formation from roots maintained in MS medium

supplemented with phytohormones (1 mg/l NAA and 1 mg/l IBA); D: Roots maintained in static MS medium.

3.1.3.4 Polyacetylenes in *C. tinctorius* callus culture

Callus culture originated from leaves of aseptically grown plants. Callus formed within three weeks, when the leaves were placed on solid MS medium supplemented with 2 mg/l NAA and 0.2 mg/l kinetin (Fig. 3.25A) (Gao et al., 2000). No callus formation was observed within three weeks, when the leaves were placed on solid MS medium supplemented with 2 mg/l 2,4-D and 2 mg/l kinetin, with 2 mg/l 2,4-D and 2 mg/l BA, or with 2 mg/l IAA and 0.2 mg/l kinetin (Fig. 3.25 B-C) (Tietjen and Matern, 1984). For identification of polyacetylenes in the callus culture, thirteen-day-old cultures were extracted and analyzed by GC-MS. No polyacetylene was detected.

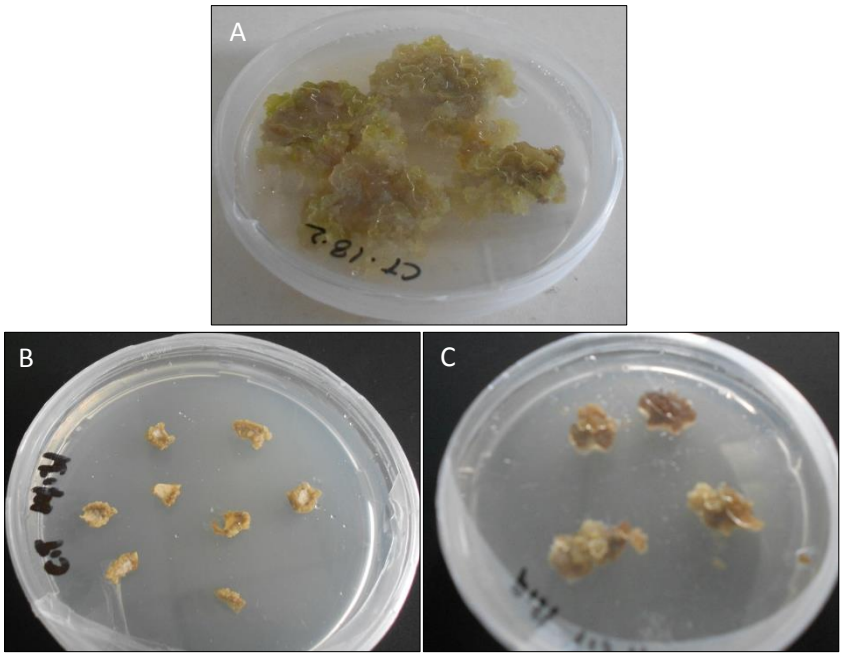

Fig. 3.25: Callus culture of *C. tinctorius*. A: Stabilized callus culture from *C. tinctorius* maintained on solid MS medium supplemented with 2 mg/l NAA and 0.2 mg/l kinetin; B: Callus maintained on solid MS medium supplemented with 2 mg/l 2,4-D and 2 mg/l BA; C: Callus maintained on solid MS medium supplemented with 2 mg/l IAA and 0.2 mg/l kinetin.

3.1.3.5 Polyacetylenes in *C. tinctoris* callus suspension culture

Callus suspension cultures were established and maintained in MS medium supplemented with 2 mg/l NAA and 0.2 mg/l kinetin. Cells of seven and thirteen-day-old cultures were harvested and their partially purified methanolic extracts were subjected to GC-MS. Only T1, 11e-3,5,7,9y was identified in seven-day-old cultures at 0.1 µmol/g DW, while no polyacetylene was identified in thirteen-day-old cultures. Before further experiments on elicitation were conducted, growth of the culture over time was studied. As shown in Fig. 3.26, a typical

growth curve was obtained with a lag phase until day 4. After the lag phase, cell cultures started to grow quickly and reached a maximum dry mass (0.69 g) on day 21. After this, no further growth took place and the cells became dark brown.

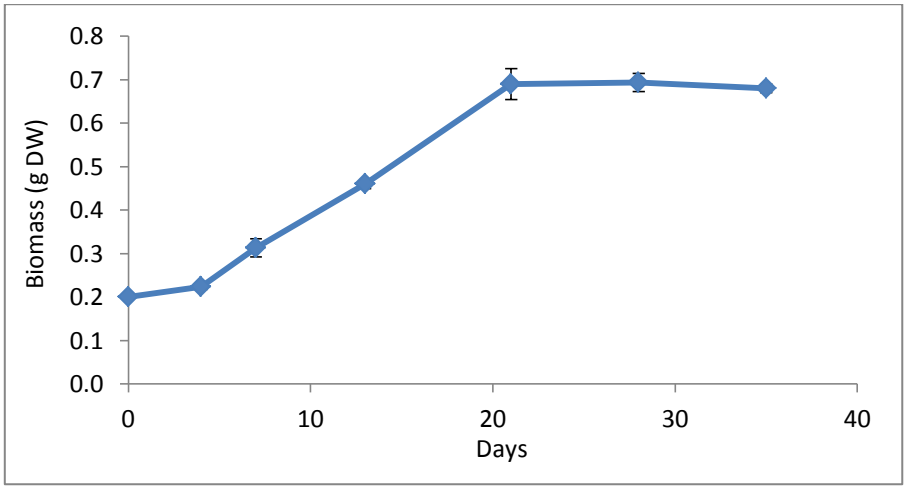

Fig. 3.26: Growth of *C. tinctorius* callus suspension cultures. Values are means ± SD of three independent experiments (n=3).

The effect of elicitors on polyacetylene content was tested by adding different concentrations of MeJ (40-100 µM), chitosan (25-300 mg/l), vanadyl sulfate (30-200 mg/l), and copper sulfate (10-50 mg/l) to seven-day-old cultures (Fig. 3.27). The incubation time with MeJ and chitosan was 72 h and with vanadyl sulfate and copper sulfate 48 h. When the callus suspension cultures were elicited with 40 µM MeJ, content of T1,11e-3,5,7,9y increased 5.4-fold as compared to control. An 1.8-fold induction was obtained with 100 mg/l chitosan. When the cultures were incubated with vanadyl sulfate and copper sulfate, no polyacetylene was detected while a minor amount of T1,11e-3,5,7,9y was detectable in the control. Taken together, callus suspension cultures of *C. tinctorius* contained only very low levels of T1,11e-3,5,7,9y, but were induced to accumulate fivefold higher levels by MeJ (40 µM added to seven-day-old cultures for 72 h). Vanadyl sulfate and copper sulfate were identified to abolish accumulation of T1,11e-3,5,7,9y in the cultures.

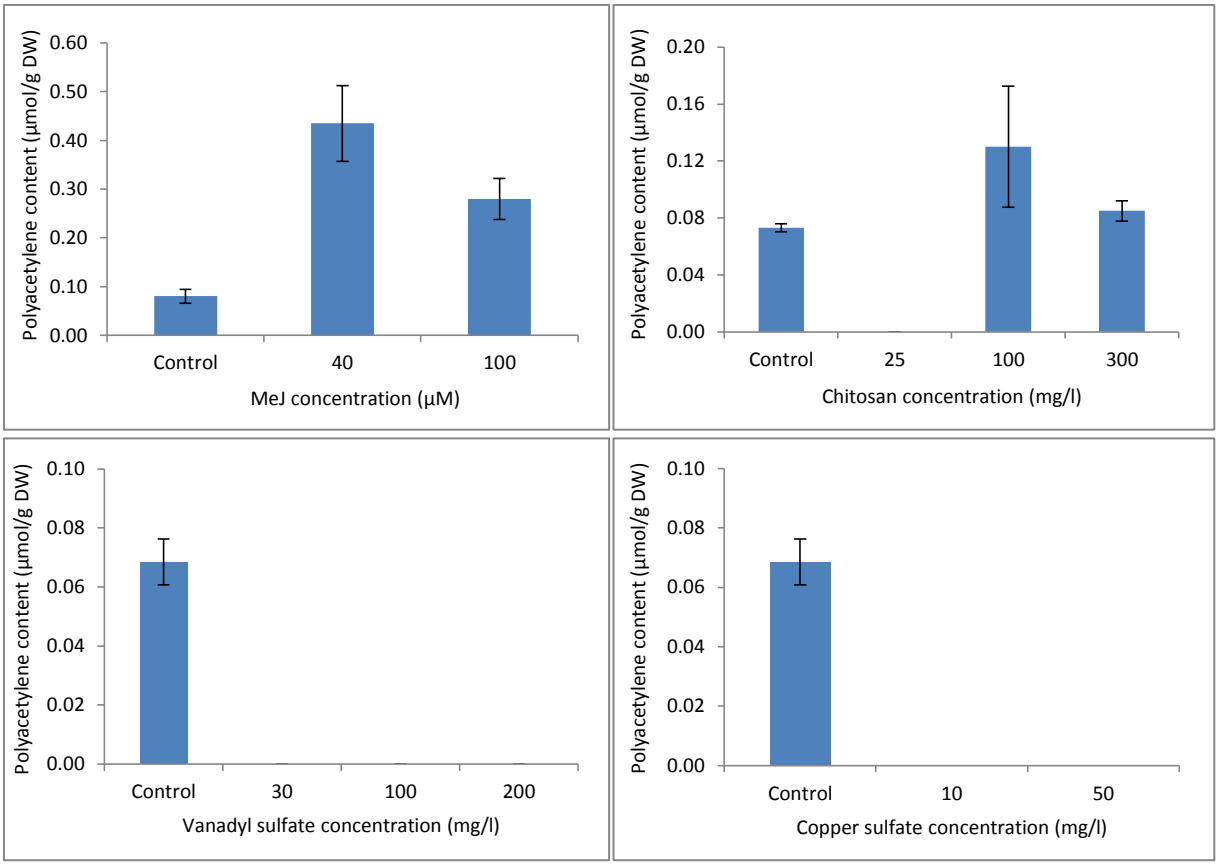

Fig. 3.27: The content of T1,11e-3,5,7,9y in seven-days-old *C. tinctorius* callus suspension cultures when challenged with different elicitors. Values are means ± SD of two independent experiments (n=2).

3.1.3.6 Polyacetylenes in *C. tinctoris* hairy root cultures

Leaf explants of aseptically grown plants were used to induce hairy roots by transformation with *A. rhizogenes* LBA 1334 and *A. rhizogenes* DSMZ 3020. Within two weeks, the leaf explants turned brown, died and no hairy root formation was observed (Fig. 3.28).

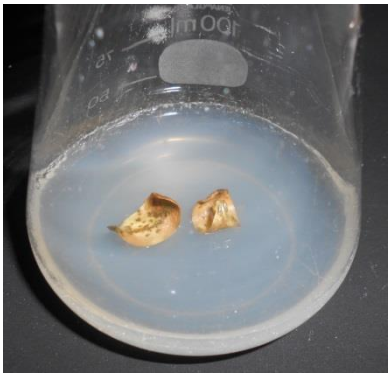

Fig. 3.28: Leaf explants of aseptically grown *C. tinctorius* two weeks after inoculation with *A. rhizogenes* LBA 1334.

3.2 Carrot callus suspension culture as transgenic production platform

3.2.1 Characterization of carrot callus suspension cultures

The growth of the suspension culture of carrot callus was studied for a period of 35 days. As shown in Fig. 3.29, the growth rate of cells was slow during the first 6 days followed by rapid growth to a maximum dry weight of 0.95 g on day 13. There was no further growth after this. From about day 21, biomass decreased and the cells became dark brown and died. In preparation of future work with carrot callus suspension culture, e.g. clonal selection, a method for protoplast isolation (Zhang et al., 2011) was tested and proved to be suitable (see Appendix 7.4).

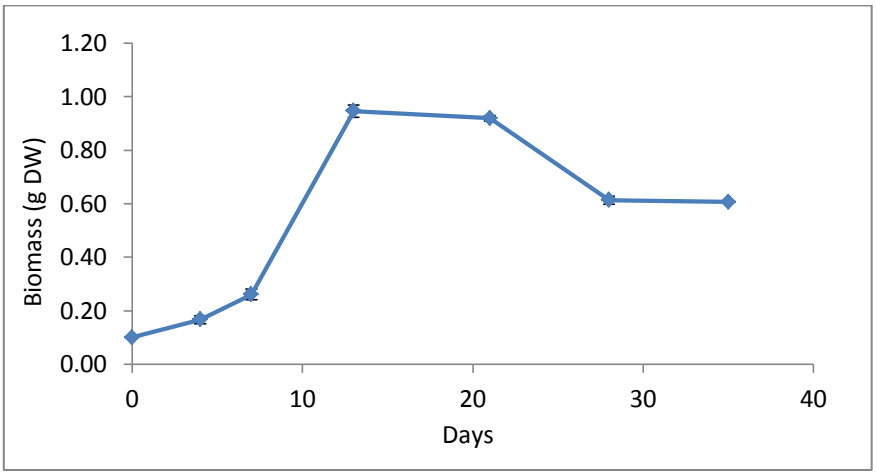

Fig. 3.29: Growth of carrot callus suspension culture. The values are the means ± SD of two independent experiments with two samples for each (n=4).

For cryopreservation of carrot suspension culture, two protocols were tested (Menges and Murray, 2004; Ogawa et al., 2012) with some modifications. We found that the cryovial, cryocontainer and the storage under the liquid nitrogen were important factors for the success of cryopreservation. The protocol according to Menges and Murray (2004) proved to be suitable as long as the cryovials were stored under liquid nitrogen (Fig. 3.30). After thawing, cells regrew within about two to three weeks. When the cryovials were stored at -80°C instead, no cell growth was observed after thawing. After the cells were cryopreserved according to Ogawa et al. (2012) no cell growth was observed even after storage under liquid nitrogen.

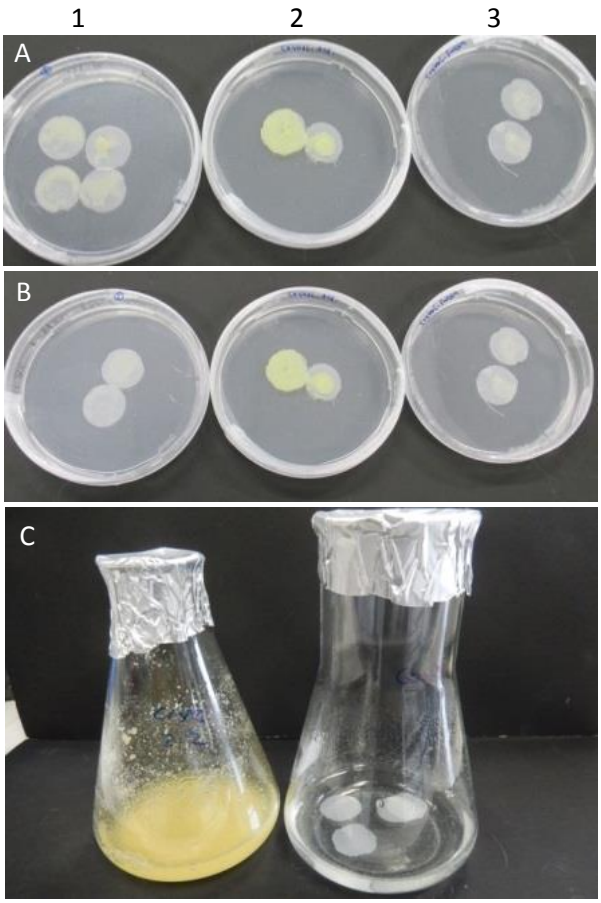

Fig. 3.30: Recovery of cells after cryopreservation of carrot callus suspension cultures. A: Cells after cryopreservation according to Menges and Murray; B: Cells after cryopreservation according to Ogawa; (1) Cryopreserved cells; (2) Non-frozen cells as positive control; (3) Cells emerged in liquid nitrogen without cryopreservation (negative control); C: Cryopreserved cells on the left and a negative control on the right after transfer to liquid medium.

The growth rate of the recovered cells was studied over a period of 35 days. As shown in Fig. 3.31, biomass of cells quickly increased and reached its maximum dry weight (0.79 g) on day 13. After this, biomass decreased and the cells became dark brown. Thus, cells after cryopreservation behaved similar to non-frozen cells, except that we did not notice a lag phase after starting the culture.

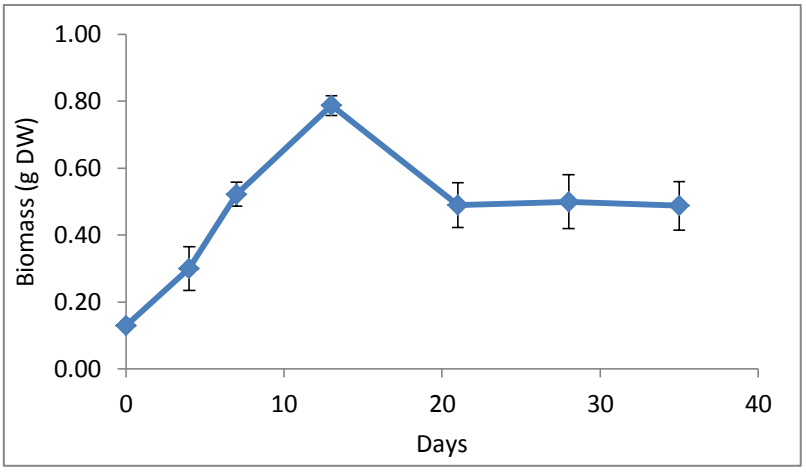

Fig. 3.31: Growth of cryopreserved carrot callus suspension culture. Shown are means ± SD of two independent experiments with two samples for each (n=4).

3.2.2 Development methods for transformation of carrot callus suspension cultures

Although methods for *A. tumefaciens*-mediated transformation of suspension cultures of callus from a variety of plants, including carrot, have been described in the literature (Hardegger and Sturm, 1998; Iantcheva et al., 2014), the transformation efficiency and practicability of the methods had to be tested under the conditions available in the Wittstock lab. Transformation efficiency was monitored in selective medium after transformation with the vector pCAMBIA1302 carrying the hygromycin resistance gene. In addition, heterologous protein expression in transgenic cells was monitored by using GFP expressed under control of the CaMV 35S promoter from pCAMBIA1302.

Method I (2.3.4.1) relied on co-cultivation of carrot callus suspension culture with *A. tumefaciens* containing pCAMBIA1302 for up to three days and subsequent selection in the suspension culture by addition of the selective antibiotic (hygromycin). After removal of the *A. tumefaciens* by addition of cefotaxime, plant cells were further cultivated with hygromycin for up to 25 days and their fluorescence was monitored. There was no fluorescence visually detectable under UV light. Cell extracts were prepared and analyzed by denaturing and non-denaturing SDS-PAGE in comparison to extracts of *E. coli* expressing GFP. While lanes with non-denatured *E. coli* extract showed a fluorescing band of 27 kDa under UV light, lanes with extracts of transformed carrot cells did not show an additional band of 27 kDa and did not show fluorescence.

Besides carrot cells from standard suspension cultures, we applied this protocol also to cells which had been synchronized with respect to their cell cycle to increase transformation efficiency according to Imani et al. (2002). However, the use of synchronized cells did not improve the results. Thus, we had to conclude that even though transformation might have

been achieved by method I, selection of transformed cells and/or heterologous protein expression did not work to a sufficient degree. Most likely, a mixture of non-transformed cells and different clones of transformed cells was obtained so that levels of heterologous protein were too low to be detected. Therefore, we tried to optimize the selection procedure and applied method II (2.3.4.2) in which droplets of callus cell suspension are distributed on solid selective medium after co-cultivation with *A. tumefaciens* containing the vector. Depending on cell density and transformation efficiency, this may allow the selection of clonal lines of transformed callus. It appeared to be easier to vary transformation conditions with this method than with method I. Therefore, upon application of method II, a number of parameters concerning the plant cells, the bacterial cells, and their co-cultivation were varied to identify conditions which result in high transformation efficiency (Tab. 3.7).

Tab. 3.7: Effects of varying conditions on transformation efficiency upon *A. tumefaciens*-mediated transformation of carrot callus suspension cultures with pCAMBIA1302 (method II). Transformation efficiency was estimated based on proportion of callus recovered on hygromycin containing medium relative to the total number of aliquots screened. +++: Many calli; ++: Few calli; +: very few calli; -: No callus.

No.	Suspension of *A. tumefaciens*		Carrot cells	Co-cultivation		Transformation efficiency
	OD_{600}	Acetosyringone (µM)		Agitation (rpm)	Duration (days)	
1	0.3	25	Synchronized	110	2	+
2	0.3	25	Synchronized	110	3	+
3	0.3	25	Synchronized	No agitation	2	-
4	0.3	25	Synchronized	No agitation	3	-
5	0.3	25	Non-synchronized	110	2	++
6	0.3	25	Non-synchronized	110	3	+
7	0.3	25	Non-synchronized	No agitation	2	-
8	0.3	25	Non-synchronized	No agitation	3	-
9	0.3	50	Synchronized	110	2	+
10	0.3	50	Synchronized	110	3	-
11	0.3	50	Synchronized	No agitation	2	-
12	0.3	50	Synchronized	No agitation	3	-
13	0.3	50	Non-synchronized	110	2	+
14	0.3	50	Non-synchronized	110	3	++
15	0.3	50	Non-synchronized	No agitation	2	-
16	0.3	50	Non-synchronized	No agitation	3	-

17	0.3	100	Synchronized	110	2	++
18	0.3	100	Synchronized	110	3	+
19	0.3	100	Synchronized	No agitation	2	-
20	0.3	100	Synchronized	No agitation	3	-
21	0.3	100	Non-synchronized	110	2	+++
22	0.3	100	Non-synchronized	110	3	++
23	0.3	100	Non-synchronized	No agitation	2	-
24	0.3	100	Non-synchronized	No agitation	3	-
25	0.8	25	Synchronized	110	2	-
26	0.8	25	Synchronized	110	3	-
27	0.8	25	Synchronized	No agitation	2	-
28	0.8	25	Synchronized	No agitation	3	-
29	0.8	25	Non-synchronized	110	2	+++
30	0.8	25	Non-synchronized	110	3	-
31	0.8	25	Non-synchronized	No agitation	2	-
32	0.8	25	Non-synchronized	No agitation	3	-
33	0.8	50	Synchronized	110	2	+
34	0.8	50	Synchronized	110	3	-
35	0.8	50	Synchronized	No agitation	2	+
36	0.8	50	Synchronized	No agitation	3	-
37	0.8	50	Non-synchronized	110	2	+
38	0.8	50	Non-synchronized	110	3	-
39	0.8	50	Non-synchronized	No agitation	2	-
40	0.8	50	Non-synchronized	No agitation	3	-
41	0.8	100	Synchronized	110	2	+
42	0.8	100	Synchronized	110	3	-
43	0.8	100	Synchronized	No agitation	2	-
44	0.8	100	Synchronized	No agitation	3	-
45	0.8	100	Non-synchronized	110	2	+++
46	0.8	100	Non-synchronized	110	3	-
47	0.8	100	Non-synchronized	No agitation	2	-
48	0.8	100	Non-synchronized	No agitation	3	-

Based on the results shown in Tab. 3.7, agitation upon co-cultivation of carrot cells with *A. tumefaciens* was most critical for transformation efficiency. Further, the experiments did not confirm the results by Imani et al. (2002), as in our hands and with other conditions ap-

plied, higher transformation efficiency was obtained with standard, i.e. non-synchronized carrot cells. The other parameters tested did not have major effects on transformation efficiency, i.e. number of hygromycin-resistant calli.

To confirm the presence of the introduced GFP in the transformed callus, the calli were converted to suspension cultures and the genomic DNA was isolated from transformed and non-transformed cells and subjected to PCR with GFP-specific primers (Fig. 3.32). This showed that only one out of three calli possessed the GFP gene. In further experiments, transformation was done using method II with parameters according to No. 5, 14, 17, 21, 22, 29, 45 in Tab. 3.7.

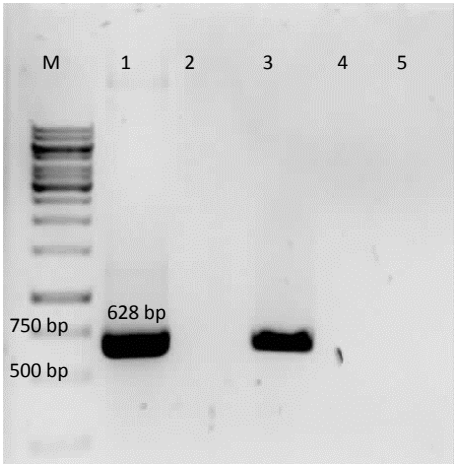

Fig. 3.32: Confirmation of T-DNA integration in hygromycin-resistant calli after transformation with pCAMBIA1302. PCR was done with primers specific for GFP. (1) PCR on plasmid (positive control); (2-4) PCR on gDNA from transformed callus; (5) PCR on gDNA from non-transformed cells. M, DNA marker (1 Kbp).

3.2.3 Transformation of carrot callus suspension cultures with a construct for expression of genes for glucosinolate biosynthesis

A construct for polycistronic expression of *Sur1*, *UGT74B1*, and *AtSOT16* (the last three genes of the glucosinolates biosynthesis pathway) under control of the CaMV 35S promoter (Geu-Flores et al., 2009b) was obtained from the lab of B. A. Halkier (University of Copenhagen). This construct has the pCAMBIA2300 backbone carrying the neomycin phosphortransferase gene (*nptII*) conferring resistance to kanamycin and is also referred to as pCAMBIA2300 + 35S::ORF2.2. The seven best conditions identified for transformation by method II (3.2.2) were applied to transform carrot callus suspension cultures with the construct. Transformants were selected on plates with 100 µg/ml kanamycin. Formation of small clumps of cell aggregates after 20 days of cultivation (Fig. 3.33) indicated that kanamycin-resistant transformants had been obtained. Transformation efficiency was monitored for the different

conditions applied (Tab. 3.8), and clumps were transferred to fresh plates with selective antibiotic. The highest transformation efficiency about 35 % was observed when the optical density of the bacterial suspension was 0.3, the culture was supplemented with 100 µM acetosyringone and the cells were co-cultivated with the bacterial suspension for three days. Besides successful selection based on kanamycin resistance, plates were free of *A. tumefaciens* upon visual inspection. Thus, cefotaxime which was routinely used in the plates to remove *A. tumefaciens*, proved to be efficient in these experiments. Cefotaxime was completely omitted from the medium after five rounds of subculture with decreasing cefotaxime concentrations.

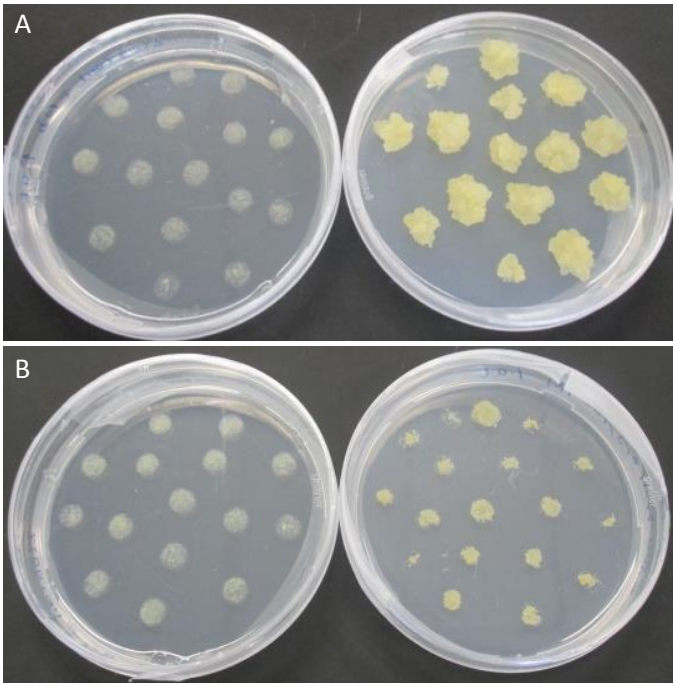

Fig. 3.33: The effectiveness of kanamycin as selective antibiotic for transformed carrot cells. A: Non-transformed cells spotted on a plate containing 100 µg/ml kanamycin (left) or on a plate without kanamycin (right); B: Transformed cells (right) and non-transformed cells (left) on a plate containing kanamycin after 20 days of incubation.

Tab. 3.8: Efficiency of *A. tumefaciens*-mediated transformation of carrot callus suspension culture with pCAMBIA2300 + 35S::ORF2.2.

OD_{600}	Acetosyringone concentration (µM)	Carrot cells	Days of co-cultivation	Transformation efficiency (%)
0.3	25	Non-synchronized	2	31
0.3	50	Non-synchronized	3	33
0.3	100	Synchronized	2	9.5
0.3	100	Non-synchronized	2	17
0.3	100	Non-synchronized	3	35
0.8	25	Non-synchronized	2	16.5
0.8	100	Non-synchronized	2	16.5

To confirm the presence of the introduced genes in the transformed callus, the calli were converted to suspension cultures and the genomic DNA was isolated from transformed and non-transformed cells. PCR was conducted on the genomic DNA to amplify a fragment of 1311 bp using primers specific for the *Sur1* gene (Fig. 3.34). A fragment of the expected size was obtained from 13 out of 16 calli analyzed.

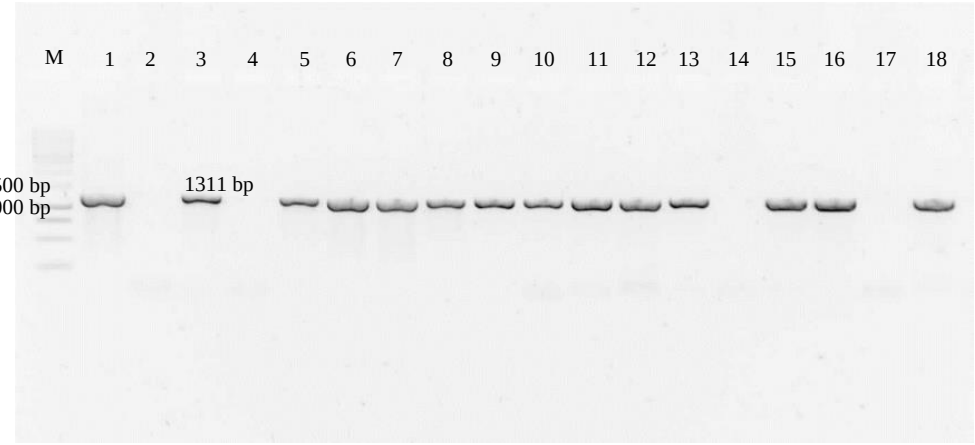

Fig. 3.34: Confirmation of T-DNA integration in kanamycin-resistant calli after transformation with pCAMBIA2300 + 35S::ORF2.2. PCR was done with primers specific for *Sur1* gene (1) PCR on plasmid (positive control); (2) PCR on gDNA from non-transformed cells; (3-18) PCR on gDNA from transformed callus. M, DNA marker (1 Kbp).

These calli were further maintained with the goal to transform them with a second construct obtained from the lab of B. A. Halkier (University of Copenhagen). This construct (also termed pLIFE7a-ORF1-GGP) was designed for polycistronic expression of *CYP79A2*, *CYP83B1*, and *GGP1* under control of the CaMV 35S promotor and carried a gene conferring Basta resistance as selectable marker (Geu-Flores et al., 2009a). Following a study by (Ganasan and Huyop, 2010; Kutty et al., 2011), tissue culture medium was supplemented with Basta (6 mg/l) for selection of transformed calli after transformation of wild type carrot suspension cultures or cultures derived from the previously obtained calli with pCAMBIA2300 + ORF 2.2.

However, in case of transformation of pLIFE7a-ORF1-GGP, growth of *A. tumefaciens* could not be stopped on the selection plates supplemented with 250 µg/ml cefotaxime (Fig 3.35). To avoid this problem, the cell suspension was plated on medium supplemented with only 250 µg/ml cefotaxime after transformation. After 9 days of incubation, the observed callus was transferred to fresh medium supplemented only with 200 µg/ml cefotaxime. After further 9 days, the callus was transferred to medium containing only 6 mg/l Basta to select the transformed cells. However, eight days after addition of Basta, bacteria growth was noticed

(Fig. 3.35). It appears that Basta and cefotaxime are incompatible selection agents under the condition used. Taken together, carrot callus transformed with pCAMBIA2300 + ORF 2.2 has been obtained. Based on initial results, 13 independent lines have been generated. However, genetic uniformness of each line, presence of all 3 genes, and their transcription has not been investigated yet. It has not been possible to transform carrot callus suspension cultures with pLIFE7a-ORF1-GGP because of an incompatibility of Basta and cefotaxime as selective agents for transformants and for removal *A. tumefaciens*, respectively. To obtain double transformed carrot callus, either the resistance marker of pLIFE7a-ORF1-GGP or the antibiotic to prevent growth of *A. tumefaciens* has to be changed.

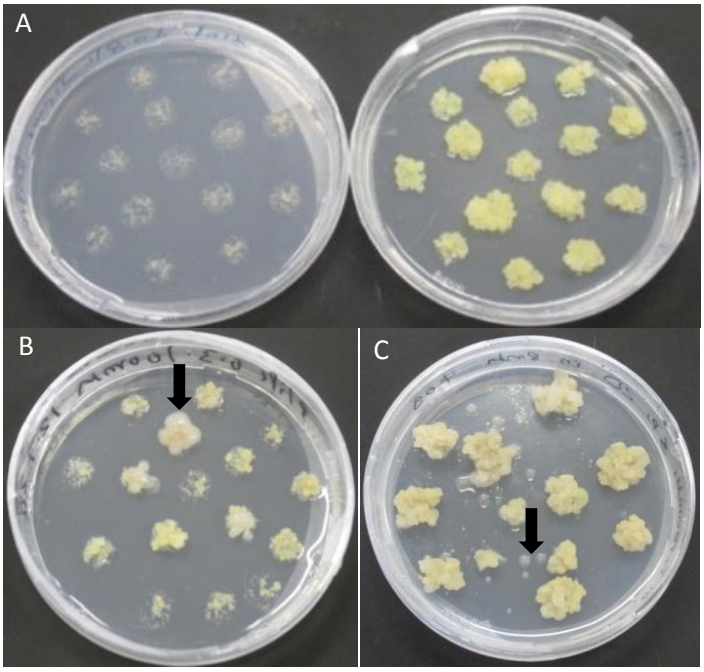

Fig. 3.35: Selection of Basta-resistant calli on plates with Basta and cefotaxime. A: Non-transformed cells spotted on a plate containing 6 mg/l Basta (left) and on plate without Basta (right); B: Bacterial growth on Basta-resistant calli after 2 weeks on a plate containing cefotaxime and Basta; C: Bacterial growth on Basta-resistant calli on a plate with Basta after two subcultures on plates with only cefotaxime. Arrows show indicate the bacterial colonies.

3.2.4 Generation of constructs for expression of myrosinase TGG1 in carrot callus suspension cultures

TGG1 is one of the six myrosinase genes found in *Arabidopsis thaliana* (Andersson et al., 2009). A plasmid construct containing the *TGG1* cDNA was used as a template to generate constructs for expression of TGG1 with a C-terminal His_6-tag (35S:TGG1-*His* tag-pPZP111) or without a tag (35S:TGG1-pPZP111). The CaMV35S promoter was introduced by a subcloning step into *Xho*I/*BamH*I-digested pRT101. Identity of the sequence was confirmed by sequencing. The expression cassette was excised from pRT101 by digestion with *Pst*I and

ligated into *Pst*I-digested pPZP111. Successful transformation of *E. coli* XL1-blue with the ligated plasmid was confirmed by PCR on plasmid DNA using gene-specific primers (see appendix (Tab. 7.1)). Vector maps of the final expression construct are shown in Fig. 3.36. The plasmids were used to transform *A. tumefaciens*. Successful transformation was confirmed by PCR with gene-specific primers after plasmid isolation from *E. coli* transformed with total DNA of transformed *A. tumefaciens*.

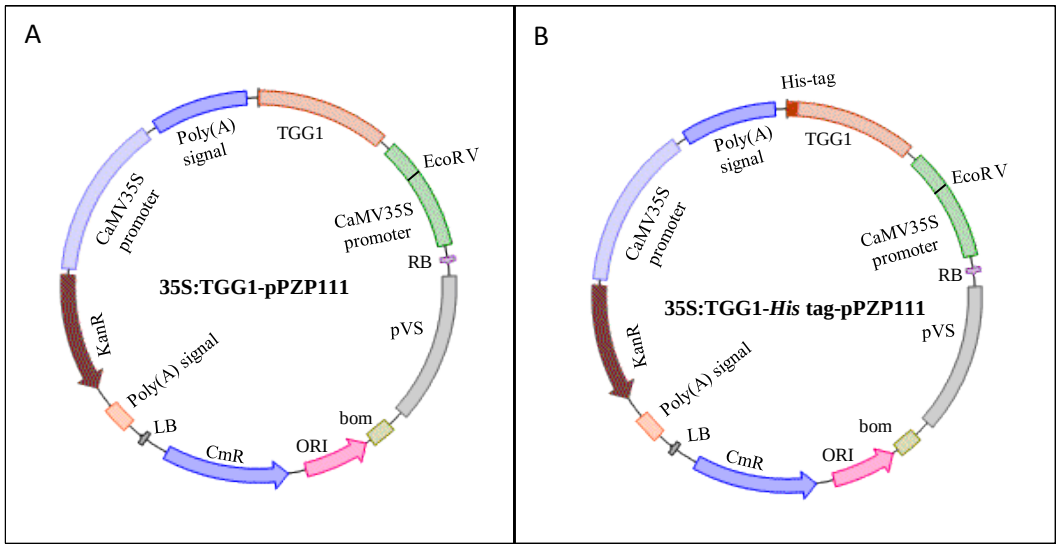

Fig. 3.36: Vector maps of expression construct for TGG1 in pPZP111. (A) 35S:TGG1-pPZP111; (B) 35S:TGG1-*His* tag-pPZP111. LB, Left border; RB, Right border.

4 Discussion

4.1 Establishing a model system for studying polyacetylene biosynthesis in Asteraceae

Polyacetylenes are considered an interesting class of special metabolites due to their structural diversity and the wide range of their biological activities (Wang et al., 2000; Kim et al., 2003; Lee et al., 2004). Polyacetylenes of the Asteraceae were in the focus of the present study. Three species were selected because of their polyacetylene profiles. *T. patula* is characterized by the existence of sulfur-containing polyacetylenes (Margl et al., 2002; Szarka et al., 2006). *A. lappa* has been reported to contain aliphatic (Takasugi et al., 1987) and sulfur-containing polyacetylenes (Washino et al., 1986), while *C. tinctorius* is characterized by the existence of only aliphatic polyacetylenes (Binder et al., 1990a). With the goal to identify a source of biosynthetically active tissue for studying polyacetylene biosynthesis, this study investigated the composition of polyacetylenes in soil-grown plants as well as in tissue cultures. There is no previous study about profiles of polycetylenes in different organs at different developmental stages in Asteraceae. Thus, different organs and developmental stages of soil-grown plants were analyzed for their polyacetylene content. Highest polyacetylene levels were found in soil-grown *T. patula* while levels in *A. lappa* and *C. tinctorius* were low.

Acetylenic thiophenes (BBT, BBTOH, BBTOAc, PBT, α-T) have previously been identified in roots, flowers, and achenes of *T. patula* (Margl et al., 2002; Szarka et al., 2006). Here, we studied several developmental stages of soil-grown plants and found the five thiophenes in all development stages (Tab. 3.2). In agreement with previous studies with flowering plants (Margl et al., 2002; Szarka et al., 2006) the level of total thiophenes was highest in roots of three-month-old plants. As in previous work with flowering plants (Margl et al., 2002; Szarka et al., 2006), BBT was the most abundant polyacetylene in roots accounting for 69 % of the total content in roots of three-month-old-plants. It accounted for 40-50 % of the total content in seedlings and roots of one-month-old plants. Polyacetylene composition in above-ground organs changed with development and depended on the organ analyzed (Fig. 3.2). For example, stems contained BBTOAc as major polyacetylene, and flower buds were rich in BBT, PBT and α-T, but lacked BBTOH and BBTOAc. In previous studies (Bicchi et al., 1992; Margl et al., 2002) flowers had high levels of PBT, slightly different to our results.

The results indicate that flower buds have some biosynthetic capacity. If the polyacetylenes would be synthesized exclusively in the roots and then be transported to the flowers, one would expect the stem to have a similar polyacetylene profile as the buds (which is not the case) (Fig. 3.2). BBTOAc, the major stem polyacetylene, might be a transport form. However, only BBT but neither BBTOH nor BBTOAc is present in the buds arguing against this hy-

pothesis. Based on the hypothetic pathway shown in Fig. 1.8, the present data suggest that flower buds possess enzymes which catalyze the addition of sulfur to triple bonds of T1e-3,5,7,9,11y to produce bi- and tri thiophenes, but lack hydroxylase and possibly acetyltransferase activity to produce BBTOH and BBTOAc. Below-ground organs seem to be a major site of BBT biosynthesis. Although BBT has been suggested as an intermediate for BBTOH and BBTOAc biosynthesis (Fig. 1.8) which are both present in below-ground organs, BBT accumulates to high levels indicating limited capacity of the downstream pathway.

Studies on polyacyetylene production in other Asteraceae plants like *A. lappa* and *C. tinctorius* are scarce. In *A. lappa*, previous studies described the presence of aliphatic and aromatic polyacetylenes in soil-grown plants (Washino et al., 1986; Takasugi et al., 1987), but the quantitative distribution of polyacetylenes among different organs at different ages and the content of polyacetylene in *in vitro* cultures have not been described before. *A. lappa* accumulated only very low levels of polyacetylenes in the growth stages analyzed, and polyacetylene profiles depended on growth stage and organ (Fig. 3.14). We did not detect aromatic polyacetylenes, but only C_{13}-aliphatic polyacetylenes (T1e-3,5,7,9,11y and T1,11e-3,5,7,9y). While seedlings did not contain detectable amounts of polyacetylene, the highest total content (ca. 0.7 µmol/g DW) was found in secondary roots of three-months-old plants. The content was, however, more than thirtyfold below the content of polyacetylenes (thiophenes) in roots of *T. patula*.

Previous studies had described the presence of aliphatic polyacetylenes in roots, flowers, stems and leaves of *C. tinctorius* (Bohlmann et al., 1966; Binder et al., 1990a). The distribution of polyacetylenes among the different organs at different ages and in *in vitro* plant tissue cultures had not been described before. Exept the stems of three-month-old plants, polyacetylenes were identified in all organs and at all developmental stages of *C. tinctorius* plants analyzed (Fig. 3.23). In agreement with previous reports (Bohlmann et al., 1966; Binder et al., 1990a), the plants accumulate C_{13}-polyacetylenes with a total of 6 double and triple bonds, namely T1e-3,5,7,9,11y, T1,3e-5,7,9,11y, T1, 11e-3,5,7,9y, T1,3,11e-5,7,9y, and T1,3,5,11e-7,9y (Fig. 3.23) (Tab. 3.6). In contrast to *T. patula* (with highest polyacetylene levels in roots) and to *A. lappa* (with no detectable polyacetylene in seedlings), the highest polyacetylene contents were found in seedlings (ca. 5 µmol/g DW) and flowers (ca. 9 µmol/g DW). Roots accumulated polyacetylenes to only about 1/10 of the content in flowers. As polyacetylene content of flowers was about tenfold above that of flower buds (Fig. 3.23), flowers are likely a major site of polyacetylene biosynthesis. It would be interesting to analyze the polyacetylene content of seeds and of germinating seeds to find out if the high polyacetylene content of

seedlings is due to de-novo biosynthesis or due to transfer and storage in seeds of polyacetylenes synthesized in flowers.

In addition to soil-grown plants, plant cell cultures can be used for studies of specialized metabolite biosynthesis. Plant cell cultures are distinguished by growing independently of geographical and seasonal influences under controlled conditions. The cultures used in this work were root cultures, hairy root cultures, callus and callus suspension cultures of *T. patula, A. lappa*, and *C. tinctorius.*

As a first step, suitable protocols for the establishment of plant tissue cultures had to be developed. Besides factors such as light and incubation period, medium composition is thought to be the most important factor for growth and development of plant tissue cultures (Gamborg et al., 1976). The medium generally consists of some or all of the following components: macroelements, microelements, vitamins, sugars as a carbon source, other organic supplements and phytohormones. Phytohormones are the most critical factor for cell growth and differentiation and also for the accumulation of secondary metabolites (Yoshikawa et al., 1986; Hara et al., 1988). Four types of phytohormone are frequently used in plant tissue cultures: auxins, cytokinins, gibberellins and abscisic acid (Johri and Mitra, 2001; Del Pozo et al., 2005). The type, concentration, and combination of phytohormones affect growth and product formation (Mantell and Smith, 1983). In this work, synthetic auxins such as 2,4-dichlorophenoxyacetic acid (2,4-D), 1-naphthylacetic acid (NAA), indole-3-butyric acid (IBA), and cytokinins such as kinetin, 6-benzylaminopurine (BAP) were used. Different tissue cultures were established for the three Asteraceae species: root cultures, hairy root cultures, callus and callus suspension cultures. Hairy root cultures grow in hormone free medium due to their ability to produce growth hormones (Shanks and Morgan, 1999), can be maintained for a long time without detectable changes in growth and are genetically stable during further subculture (Hu and Du, 2006). Hairy root cultures consist of different cell types and cells in different physiological states. The specific cell types at defined stages in their development may be responsible for production of a particular metabolite as only part of the cells will be productive at any specific stages (Walton et al., 1999). Hairy roots of *T. patula* showed high growth rates and branching as observed in hairy roots derived from other plant species, such as *Ambrosia maritima* (Zid and Orihara, 2005) and *Lobelia cardinalis* (Yamanaka et al., 1996). All obtained hairy root lines from *T. patula* had nearly the same morphology. Hairy root cultures could serve as an alternative to the root cultures for *A. lappa* and *C. tinctorius*. However, hairy roots have not been obtained when leaf explants of aseptically-grown plants were transformed with two different *A. rhizogenes* strains. This may have

been due to resistance of the explants to the used strains due to presence of certain factors which have antibacterial activities. Root cultures of *A. lappa* have not been established previously for studying of polyacetylenes or other secondary metabolites. Unintended formation of callus hampered the work with root cultures. Although addition of 0.2 mg/l NAA to MS medium prevented the formation of callus, the root cultures grew very slowly. Polyacetylene composition in tissue cultures of *C. tinctorius* has not been described previously. A root culture from *C. tinctorius* could not be established in several trails (3.1.3.3) although the establishment of the root culture from *C. tinctorius* has been achieved previously (Bernard et al., 2011). Callus suspension cultures and callus cultures are undifferentiated and totipotent cells. Callus forms naturally on plants in response to wounding or invasion. In all species, supplementation with a mixture of auxins and cytokinins was essential for callus induction and led to good callus formation which allowed subsequent conversion to callus suspension cultures.

In *T. patula*, root and hairy roots grew well without addition of phytohormones. The biomass reached a maximum of 0.60 g DW on day 28 for root cultures and of 0.57 g DW on day 28 or 0.49 g DW on day 13 for hairy root lines 2 and 3, respectively. Thus, the root and hairy root cultures had approximately the same growth rate. Despite this, hairy roots are generally preferred because of their genetic stability (Agostini et al., 2013) and easier maintenance over longer periods. In *A. lappa*, the callus suspension cultures grew faster than the root cultures with a maximum biomass of 0.92 g DW reached on day 21. In *C. tinctorius*, the callus suspension cultures had the highest growth rate with a maximum biomass of 0.69 g DW on day 21. Taken together, sufficient biomass has been obtained from tissue cultures of all three species within growing periods between 13 and 28 days.

For *T. patula*, most promising results from tissue cultures experiments were obtained with root and hairy root cultures. Confirming previous reports (Mukundan and Hjortso, 1990; Margl et al., 2002), the *in vitro* root cultures of *T. patula* contained the same thiophene pattern as soil-grown plants. Thiophene concentrations in the root cultures were about three to foufold higher than in roots of three-month-old plants. This was similar to a previous report (Margl et al., 2002) in which thiophene concentrations in the root culture of *T. patula* were about twofold higher than the concentration in intact plants. Despite polyacetylene production in root cultures, the increase of polyacetylene content after subculture was not very steep. In the root culture of *A. lappa* two aliphatic polyacetylenes T1e-3,5,7,9,11y and T1,11e-3,5,7,9y were identified which were the same as those found in the soil-grown plants, but total amount was very low. Although the growth of the root culture was slow, 100 µM MeJ and 100 mg/l chitosan were used to test elicitation. 100 mg/l chitosan increased the total amount of polya-

cetylenes about 2.2-fold compared to the control. Future studies should test other concentrations and time points to increase the elicitation effects. However, slow growth will limit the use of *A. lappa* root culture for studies the biosynthesis of polyacetylenes.

Hairy root cultures are considered the most important system for studying specialized metabolites, as they often produce the same or similar secondary compounds as root cultures or soil-grown plants (Margl et al., 2002; Szarka et al., 2006). As hairy root cultures of *T. patula* were easier to maintain and accumulated thiophenes to about the same level, elicitation experiments were only done with hairy root cultures. Enhanced growth and branching in these cultures is due to expression of *rol* genes (*rolA, rolB* and *rolC*). This does not only affect the inherent control system responsible for growth (Walton et al., 1999) but also affects production of specialized metabolites with up to more than 100-fold increase in hairy roots as compared to root cultures (Kiselev et al., 2007; Bulgakov, 2008). Hairy root lines 2 and 3 were chosen for further studies because they had better growth rate than the others. Variation in growth rate and total thiophene content were observed in the two lines (Fig. 3.8). This variation could be a consequence of the T-DNA insertion at different sites in the genome. The production of specialized metabolites in tissue cultures can be uplifted by using elicitors. Elicitors may increase the production of specialized metabolites by stimulating signaling pathways resulting in activation of genes involved in their biosynthesis (Goossens et al., 2003). In this work, the elicitation of thiophenes was tested by using MeJ, chitosan, vanadyl sulfate and copper sulfate. The use of vanadyl sulfate to induce the thiophene production in the hairy roots of *T. patula* has been described previously. Hjortso and Mukundan (1994) found that vanadyl sulfate induces the production of thiophenes in hairy roots of *T. patula* to about two-fold. Besides, vanadyl sulfate induced the thiophene-related compound thiarubine A of *Ambrosia artemisiifolia* eightfold compared to control (Bhagwath and Hjortsø, 2000). The elicitation of thiophenes by using MeJ and chitosan has not been tested before. The two hairy root lines differed in their response to elicitors. The highest induction factor was obtained upon 72 h incubation with 100 µM MeJ in line 2 and with 30 mg/l vanadyl sulfate in line 3 when elicitors were added in the middle of the growth phase (day 21 for line 2, day 7 for line 3) (Fig. 3.12). Thus line 3 seems to be most suitable for further investigation.

Callus suspension cultures are generally characterized by a rapid growth rate and often used for investigation of specialized metabolite biosynthesis. Although callus cultures from *A. lappa* and *C. tinctorius* were established, the callus cultures did not produce detectable amounts of polyacetylenes. In the callus of *T. patula*, thiophenes like BBT, PBT and α-T were detected in small amounts (about 1.5 µmol/g DW) whereas no hydroxylated or acetylated

BBT derivatives were detected (Fig. 3.5). BBT was the major thiophene accounting for 60 % of the total content. While previous studies found BBTOAc in callus cultures of *T. patula* and *T. mendocina* (Benavides and Caso, 1993; Margl et al., 2002), the absence of BBTOH was in agreement with previous findings (Benavides and Caso, 1993; Margl et al., 2002). As the callus cultures contained 50-100 times less thiophenes than root cultures, we do not consider them a good system for further biosynthesis studies.

Callus suspension cultures of *A. lappa* did not contain detectable amount of polyacetylene. Therefore, we tried to induce biosynthesis by elicitor addition and compared polyacetylene content and profile in callus suspension cultures of *A. lappa* and *C. tinctorius* with and without addition of elicitors. Addition of elicitors to *A. lappa* callus suspension cultures did not affect polyacetylene content (3.1.2.5). The inability to produce polyacetylenes even after elicitation indicates that the *A. lappa* suspension culture is not a good system for studying polyacetylene biosynthesis. However, other elicitor could be tested. In callus suspension cultures of *C. tinctorius*, one polyacetylene (T1,11e-3,5,7,9y) was detected at low levels when the cells were seven days old whereas no polyacetylene was detected in 13-day-old suspension cultures. A fivefold induction of T1,11e-3,5,7,9y was obtained upon addition of 40 µM MeJ (Fig. 3.27). Thus, callus suspension cultures of *C. tinctorius* are a promising model for further biosynthesis studies. Chitosan also induced biosynthesis, but to lesser extend (< 2-fold). When the cultures were incubated with vanadyl sulfate and copper sulfate, no polyacetylene was detected while untreated controls contained minor amounts of polyacetylenes. Thus, these compounds appear to interfere with either biosynthesis or storage of polyacetylenes or induce their degradation. This was an unexpected result. However, it revealed that vanadyl sulfate and copper sulfate could be used as tools to inhibit polyacetylene accumulation. Future studies could test the effect of a combined treatment with MeJ, vanadyl sulfate and copper sulfate. More research is needed to find out, how the two compounds affect polyacetylene accumulation.

Taken together, this study established some model systems to study the biosynthesis of polyacetylenes in soil-grown plants and tissue cultures. Although it was previously confirmed that polyacetylenes originate from fatty acids (Bohlmann et al., 1973; Minto and Blacklock, 2008), there is very little known about the enzymes involved. We aimed at establishing the analytical methods and the plant model systems for further studies on the biosynthesis of thiophenes and polyacetylenes in Asteraceae. Future studies should address the sequence of biosynthetic reactions, the localization of enzyme activities in the plant, and the identity of the enzymes. For example, seedlings of *C. tinctorius* could be fed with oleic acid carrying a ^{13}C-

label at C_{18}, C_{16}, C_{14} or C_{12} to test for biosynthetic activity and to see which carbon atoms are introduced into T1e-3,5,7,9,11y. Analysis of differential gene expression in *C. tinctorius* callus suspension culture with and without addition of 40 µM MeJ could aid in identification of acetylenase genes.

T1e-3,5,7,9,11y which is the precursor for thiophene biosynthesis according to the previous labeling experiments (Bohlmann and Hinz, 1965; Bohlmann et al., 1966) can be isolated from the flowers of *C. tinctorius*. The isolated T1e-3,5,7,9,11y could be used to investigate the sulfur donor e.g. cysteine or glutathione in biosynthesis of thiophenes in *T. patula* using hairy roots of *T. patula*. The compound can also be used in enzyme assays to detect enzymes catalyzing sulfur addition or acetylenases, respectively. T1,11e-3,5,7,9y is likely to be the direct precursor of sulfur-containing polyacetylenes (Fig. 1.8) and would be a valuable compound for further experiments. However, although T1,11e-3,5,7,9y is elicited fivefold in *C. tinctorius* callus suspension cultures by 40 µM MeJ it would not be feasible to isolate the compound from the cultures for further studies due to the low absolute amount.

4.2 Carrot callus suspension cultures as heterologous host for glucosinolate biosynthesis

Glucosinolates are pharmaceutically and agriculturally interesting due to the biological activities of their breakdown products such as cancer-preventive (Talalay and Fahey, 2001), antibacterial (Aires et al., 2009), nematicidal, and insecticidal activity (Wittstock et al., 2003). A number of glucosinolates is commercially available. Their production is based on isolation from plant material. The isolation procedure is rather simple if the desired glucosinolate occurs as major or single glucosinolate in an easy-to-grow plant. However, compounds like glucoraphanin, the precursor of the anti-carcinogenic sulforaphane, are typically components of glucosinolate mixtures, which makes their isolation more difficult. This difficulty could be overcome by biotechnological production of individual glucosinolates in a heterologous host. The goal of the present study was to test, if plant suspension cultures could be used as heterologous host for glucosinolate production. Plant cells were chosen as they provide all compartments required for housing the complex pathway of aliphatic glucosinolate biosynthesis i.e. plastids and endoplasmic reticulum. Suspension cultures are a scalable system, and controlled conditions with optimized medium compositions might further exogeneous metabolite production. Carrot callus suspension cultures were chosen because they were already availabe in the Wittstock lab. In addition they are already in use for commercial production of therapeutic proteins (Paul and Ma, 2011). Benzylglucosinolate was chosen as a model glucosinolate for proof of principle because of its relatively simple biosynthetic pathway which can be accomplished by transfer of only six genes.

Transfer of glucosinolate biosynthesis, especially benzylglucosinolate, to non-cruciferous plants was previously obtained by transferring the six biosynthetic genes into tobacco by using two expression constructs each possessing a 2A polycistronic ORF under control of the CaMV35S promoter (Geu-Flores et al., 2009a). The same expression constructs were used in the present study in the hope that they would allow strong enough expression for poof of principle, but prevent gene silencing.

Transformation of suspension cultures from a variety of plants, including carrot, has been described before (Hardegger and Sturm, 1998; Iantcheva et al., 2014). Two methods for *A. tumefaciens*-mediated transformation were tested using pCAMBIA1302 for expression of GFP under control of the CaMV35S promoter (3.2.2). Method I relied on transformation of suspension cultures without conversion to calli. The selection of transformed cells and /or heterologous protein expression did not work to a sufficient degree. The use of synchronized cells according to Imani et al. (2002) did not improve the results. Method II relied on the generation of calli for selection. After optimization of parameters concerning the plant cells, the *A. tumefaciens* cells, and their co-cultivation, this method was successfully applied. Based on this and in agreement with previous results (Iantcheva et al., 2014) obtained with *Medicago truncatula* cell suspension culture transformation, agitation during co-cultivation of plant and bacterial cells was the most critical factor for a sufficient transformation efficiency.

A construct for polycistronic expression of Sur1, UGT74B1, and AtSOT16 (the last three genes of the benzylglucosinolate biosynthesis pathway), also referred to as pCAMBIA2300 + 35S::ORF2.2, was transferred to carrot suspension culture. This construct carries neomycin phosphotransferase gene (NPTII) which inactivates kanamycin by phosphorylation. As the concentration of the selective antibiotic is a critical factor for efficient selection of transformed calli, the optimum kanamycin concentration was experimentally determined. While 300 µg/ml were reported to be best in the literature (Wurtele and Bulka, 1989), 100 µg/ml worked best in the present study. May be the best concentration of the selective antibiotic depends not only on the plant species but also on the cultivar. The highest transformation efficiency (about 35 %) was observed when the optical density of the bacterial suspension was 0.3, when it was supplemented with 100 µM acetosyringone, and the plant cells were co-cultivated with the bacterial suspension for three days. Kanamycin-resistant callus was converted to suspension culture and tested for presence of the transgene by PCR. Besides successfully transformed calli, this revealed a number of false positive, i.e. kanamycin-resistant calli with no detectable integration of ORF2.2 indicating that further optimization of the selection protocol could be advantageous.

A construct for polycistronic expression of CYP79A2, CYP83B1, and GGP1 (the first three genes of the glucosinolates biosynthesis pathway), also referred to as pLIFE7a-ORF1-GGP, was separately transferred to carrot suspension culture. This construct carried the phosphinothricin resistance gene (*Bar* gene) as a selectable marker gene for selection of transformed plant cells. Although phosphinothricin is most commonly used as a selection agent for soil-grown plants, it has been used previously for transgene selection in *in vitro* cultures (Ganasan and Huyop, 2010; Kutty et al., 2011). Phosphinothricin inhibits glutamine synthetase which leads to accumulation of intracellular ammonia followed by cell death. The enzyme phosphinothricin acetyltransferase encoded by the *Bar* gene catalyzes acetylation of phosphinothricin resulting in its detoxification (Thompson et al., 1987). Among the different concentrations of phosphinothricin that we have tested, 6 mg/l was chosen as a minimal stringent concentration for selection of transgenic cells. Non-transformed cells did not grow on media with 6 mg/l phosphinothricin. Cefotaxime was also added to the medium to stop *A. tumefaciens* growth. However, when pLIFE7a-ORF1-GGP was introduced to the cells, growth of *A. tumefaciens* could not be stopped on the selection plates although appropriate concentrations of cefotaxime were added. Thus, we were not able to select transgenic calli carrying ORF1. We assume that cefotaxime is not compatible with phosphinothricin or phosphinothricin acetyltransferase. In future experiments either phosphinothricin-resistance should be exchanged by another selection marker or *A. tumefaciens* should be removed by different antibiotics (timentin or carbenicillin).

Cell suspension cultures are valuable hosts for genetic engineering due to the high growth rate and the possibility for scale-up. However, the routine subculturing may lead to contamination and may reduce transgene stability. Therefore, we tested if cryopreservation can be applied to reduce the routine subculturing, to minimize the chance of microbial contamination and to maintain master cell banks. Cryopreservation techniques used to preserve plant cells are either vitrification-based techniques or classical methods. Vitrification is also called fast cooling cryopreservation because it involves rapid cooling of plant cells by immersion in liquid nitrogen (Sakai et al., 1990). The classical technique depends on simple dehydration of plant cells before immersion in liquid nitrogen to avoid ice crystal formation inside the cells, and this is achieved by a slow cooling rate. A slow cooling rate leads to the formation of extracellular ice before formation of intracellular ice which causes flow of water out of the cells (Engelmann, 2004). Cryoprotectants are added to reduce cell injuries during freezing as they reduce cell size and prevent the formation of ice inside the cells (Chian and Quinn, 2010). Cryoprotectants are classified depending on their ability to permeate into the cell membrane

(Chian and Quinn, 2010). Non-permeating protectants such as sugars and sugar alcohols increase the osmotic pressure and decrease the cell size (Chian and Quinn, 2010). Permeating protectants such as DMSO and glycerol rapidly penetrate into the cell membrane and prevent cell damage during freezing by replacing the most of intracellular water. Besides, permeating protectants decrease the concentration of solute of the remaining water inside the cells by working as solvent and hence decrease the stress induced by salt (Chian and Quinn, 2010). Cryoprotectants are most effective when used in combination rather than alone (Jain et al., 1996; Cho et al., 2000). The thawing of cells after classical cryopreservation or vitrification should be as rapid as possible to prevent recrystallization (Reinhoud et al., 2000). In this work, the classical technique with sorbitol and DMSO as cryoprotectans was used to preserve the carrot cell suspension culture. The protocol was transferred from (Menges and Murray, 2004) with some modifications. The use of proper cryovials, a cryocontainer, and storage in liquid nitrogen were important factors for the success of cryopreservation. The recovery of cryopreserved cells was determined by studying the growth rate of the recovered cells as compared to control cells. Viability test such as the triphenyltetrazolium chloride (TTC) reduction method might be used in the future to evaluate survival rates of cryopreserved cells. This test has been previously been applied to cryopreserved plant suspension cultures (Shibli et al., 2001; Mikuła et al., 2011).

Taken together, this study established some basic techniques required for use of carrot callus suspension cultures for heterologous production of glucosinolates. We aimed at transferring benzylglucosinolate biosynthesis to the plant cell suspension cultures for proof of principle before transfer of glucosinolates with more complex biosynthesis such as glucoraphanin. Benzylglucosinolates production requires transformation with two constructs. Thus, combinations of different selectable markers for transgenic plant cells and *A. tumefaciens* have to be tested. Next, the two constructs (pLIFE7a-ORF1-GGP and pCAMBIA2300 + 35S::ORF2.2) will be transferred to the carrot suspension cultures together in one step to produce benzylglucosinolate. Successful transformation will be checked by PCR. Formation of benzylglucosinolate will be tested by LC-MS. If benzylglucosinolates biosynthesis was successfully transferred to carrot callus suspension cultures, further experiments will include clonal selection through conversion to protoplasts, medium optimization and precursor (phenylalanine, sulfate) feeding to increase production, optimization of fermentation conditions and scale-up. Although CaMV35S promoter has been previously used to control heterologous protein expression in carrot cell suspensions (Shaaltiel et al., 2007), other promoters will be tested with the goal to maintain transgene stability. For example, a weaker constitutive promoter such as

tCUP1 promoter from tobacco and a strong inducible promoter such as alcA from *Aspergillus nidulans* along with alcR transcriptional activator which mediate the transcription induction in the presence of ethanol (Hemmati and Basu, 2015) will be tested.

Glucosinolate production in transgenic plant cell suspension cultures could also be tested in other species to either increase growth rate (tobacco BY2 cells) or to make it easier to study effects of transgene expression on host gene transcription and metabolism of host cells. A species with a sequenced genome but no endogenous glucosinolates (e.g *Lotus japonicus*) could be used.

5 Summary

Plant specialized metabolism is a rich source of bioactive compounds for pharmaceutical use. To exploit the potential of structurally diverse specialized metabolites produced by plants, they must be available in sufficient amounts for pharmacological and clinical studies. Metabolic engineering represents one possibility for biotechnological production of specialized metabolites. However, it requires that the biosynthetic pathway for the compound of interest has been completely elucidated and the responsible genes are known. The present study addressed the biosynthesis of two different classes of specialized metabolites, the polyacetylenes and the glucosinolates.

Polyacetylenes are specialized metabolites characterized by having double and C-C triple bonds. They are found in higher plants, commonly in Asteraceae, Apiaceaea and Araliaceae, as well as in fungi, algae and sponges. Enzymes involved in polyacetylene biosynthesis are largely unknown. One aim of this work was to establish analytical methods and plant model systems for studying polyacetylene biosynthesis. Three species of Asteraceae were chosen for these investigation: *Tagetes patula*, *Arctium lappa*, and *Carthamus tinctorius.* Thiophenes and polyacetylenes in soil-grown plants and tissue cultures were identified by GC-MS and quantified by GC-FID. We found thiophenes in *T. patula* and C_{13}-polyacytelenes in *A. lappa* and *C. tinctorius*.

In soil-grown *T. patula* plants, above- and below-ground organs at different developmental stages contained five different thiophenes. The root was identified as a rich source of butenynyl-bithiophene with highest levels in roots of three-month-old plants (ca. 20 µmol/g DW). In *A. lappa*, C_{13}-polyacetylenes were identified in one-month-old plants and secondary roots of three-month-old plants, but only at very low levels. No polyacetylene was identified in seedlings, leaves and primary roots of three-month-old plants. In *C. tinctorius,* C_{13}-polyacetylenes were present in above- and below-ground organs at different developmental stages. Seedlings and flowers contained the highest levels of C_{13}-polyacetylenes (ca. 5 µmol/g DW and ca. 9 µmol/g DW respectively). Two compounds with three and two acetylenic bonds predominantly accumulated in seedlings, while a mixture of penta-, tetra-, and triynes accumulated in flowers. The level of C_{13}-polyacetylenes in flowers was about six times higher than that in roots of three-month-old plants.

Root cultures and hairy roots from *T. patula* had a good growth rate and contained the same thiophenes as the soil-grown plants. They accumulated an about three to fourfold higher concentration of thiophenes (ca. 85 µmol/g DW) than roots of three-month-old plants. Hairy roots were used for elicitation experiments because they are much easier to maintain over

long periods. Upon elicitation of two hairy root lines (Line 2, Line 3) with 100 µM MeJ or 30 mg/l vanadyl sulfate, thiophenes increased about twofold compared to the controls. Callus cultures of *T. patula* had only very low levels of three thiophenes and were not used for further experiments. Root cultures of *A. lappa* contained the same polyacetylenes as soil-grown plants, but at very low levels. Addition of 100 mg/l chitosan led to an about twofold increase of the total polyacetylene content. Because of small biomass production, the root cultures of *A. lappa* are not suitable for further studies. There was no polyacetylene detectable in callus and suspension cultures even after elicitation. Hairy root cultures could not be established. In case of *C. tinctorius*, only callus and suspension cultures were established successfully. Although the callus of *C. tinctorius* contained no polyacetylene, 1,11-tridecadiene-3,5,7,9-tetrayne was identified in callus suspension cultures and increased about fivefold when cultures were elicited with 40 µM MeJ. The accumulation of 1,11-tridecadiene-3,5,7,9-tetrayne was abolished by addition of vanadyl sulfate or copper sulfate.

The results provide valuable insight for future studies of the biosynthesis of thiophenes and C_{13}-polyacetylenes in Asteraceae. For example, flowers of *C. tinctorius* can be used to isolate 1-tridecene-3,5,7,9,11-pentayne, the precursor of thiophenes. 1-Tridecene-3,5,7,9,11-pentayne can be used as a substrate to study the formation of the thiophene ring system in *T. patula* hairy root cultures. *C. tinctorius* callus suspension cultures with and without addition of 40 µM MeJ could be subjected to differential gene expression analysis to identify candidate acetylenase genes.

Glucosinolates are sulfur- and nitrogen-containing plant specialized metabolites found within the order *Brassicales*, which includes agriculturally important crops of the Brassicaceae family such as oilseed rape. The complete pathway for benzylglucosinolate biosynthesis is known. To enable transfer of benzylglucosinolate biosynthesis to transgenic plant cell suspension cultures to test the suitability of such cultures as production platform, this work established *A. tumefaciens*-mediated transformation and cryopreservation methods for carrot callus suspension cultures.

As a result, co-cultivation of plant cells with *A. tumefaciens* and clonal selection on solid medium resulted in transformation efficiency of up to 35 %. Specifically, a maximum transformation efficiency of about 35 % was observed under the following condition: 100 µM acetosyringone, bacterial suspension with OD_{600} of 0.3, co-cultivation period of 3 days. Using this optimized method, a polycistronic construct of the last three genes of benzylglucosinolate biosynthesis was successfully transferred to carrot cells. Transformation with a second polycistronic construct containing the first three genes for benzylglucosinolate biosynthesis was

apparently hampered by incompatibility of the selective agent for transformants (Basta) and the antibiotic used to delete *A. tumefaciens* (cefotaxime). In future studies, alternative antibiotics should be applied to avoid this incompatibility. Once double transformed lines are obtained, they will be screened for benzylglucosinolate production. Benzylglucosinolate producing lines will be used to maximize yield by optimizing fermentation conditions in a bioreactor. If successful, this could provide a basis for biotechnological production of diverse glucosinolates and other plant specialized metabolites in a scalable plant system.

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7 Appendix

7.1 Primers

Tab. 7.1: Primers used for PCR and sequencing. Primers were obtained from Invitrogen (Life Technologies™).

No.	Label	Sequence (5' to 3')
1	*Xho*I-TGG1 Fw	CTT ACT CGA GAT GAA GCT TCT TAT GCT CGC
2	TGG1-*BamH*I Re	GAA TGG ATC CTC ATG CAT CTG CAA GAC TC
3	TGG1-C-Terminal *His* tag-*BamH*I Re	GAA TGG ATC CTC AGT GGT GGT GAT GGT GAT GAT GGT GGT GAT GTG CAT CTG CAA GAC TCT TC
4	CYP83B1 Fw	ATG GAT CTC TTA TTG ATT ATA GCC GG
5	CYP83B1 Re	TCA GAT GTG TTT CGT TGG TGC
6	SUR1 Fw	ATG AGC GAA GAA CAA CCA CAC G
7	SUR1 Re	TCA AAC CCA GAG CAT CCC CTG
8	*rolB* Fw	TACTGCAGCAGGCTTCATGCA
9	*rolB* Re	GCTTTCCCGACCAGAGACTG
10	35S Prom Fw	CCC ACT ATC CTT CGC AAG ACC C
11	35S Term Re	GAG ATA GAT TTG TAG AGA GAG ACT GGT G
12	T7 Fw	TAA TAC GAC TCA CTA TAG GG
13	T7 Term	GCT AGT TAT TGC TCA GCG G
14	TGG1-Intern Fw	ATT CTC CAT TGC GTG GTC AAG

7.2 Sequence of TGG1 cloned in pRT101

7.2.1 35S TGG1-pRT101

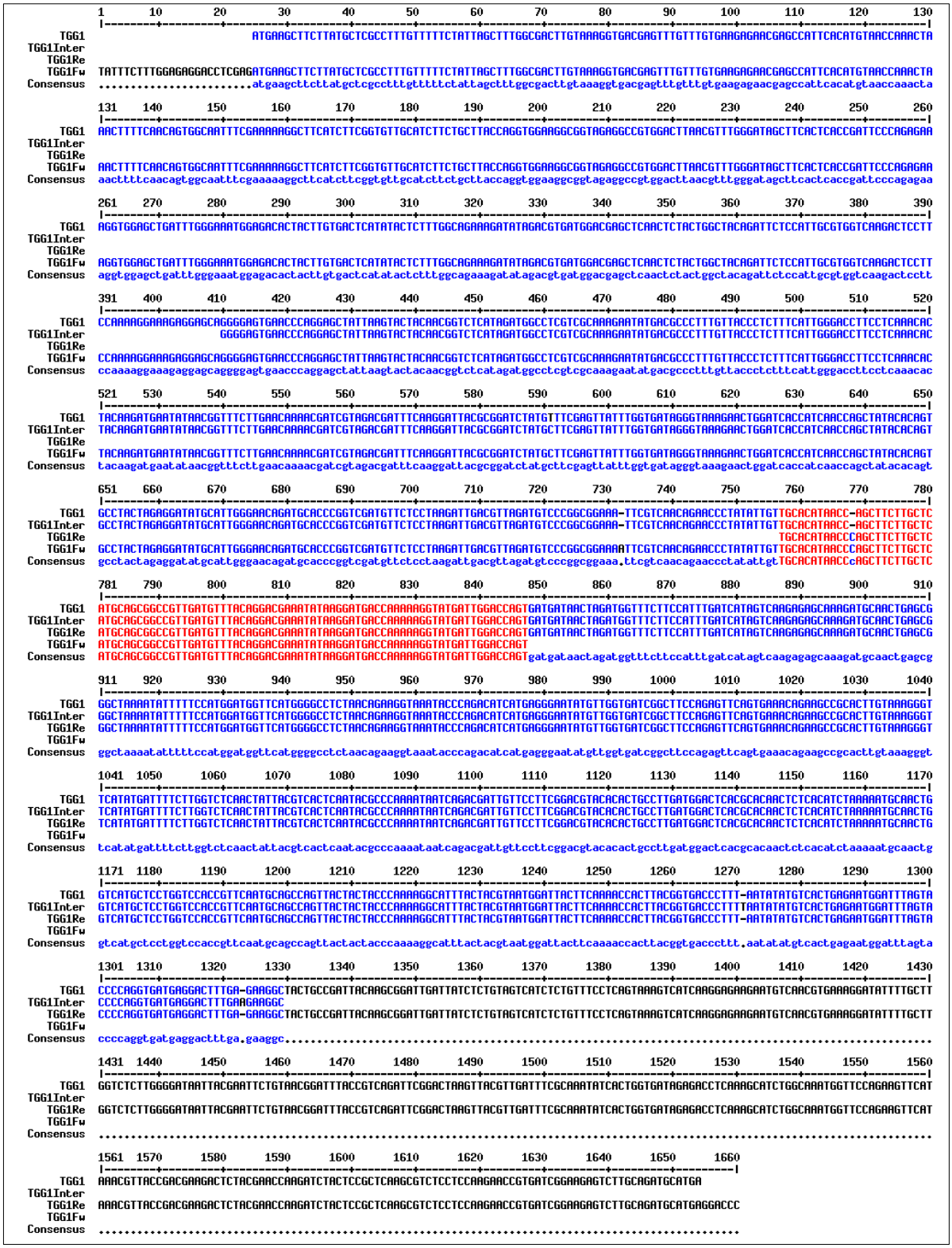

Fig. 7.1: Comparison of nucleotide sequence between TGG1 from Genbank and TGG1 cloned in pRT101. TGG1: nucleotide sequence from data bank; TGG1Int: sequence by using internal primer; TGG1Re: sequence by using reverse primer; TGG1Re: sequence by using forward primer.

7.2.2 35S TGG1-*His* tag-pRT101

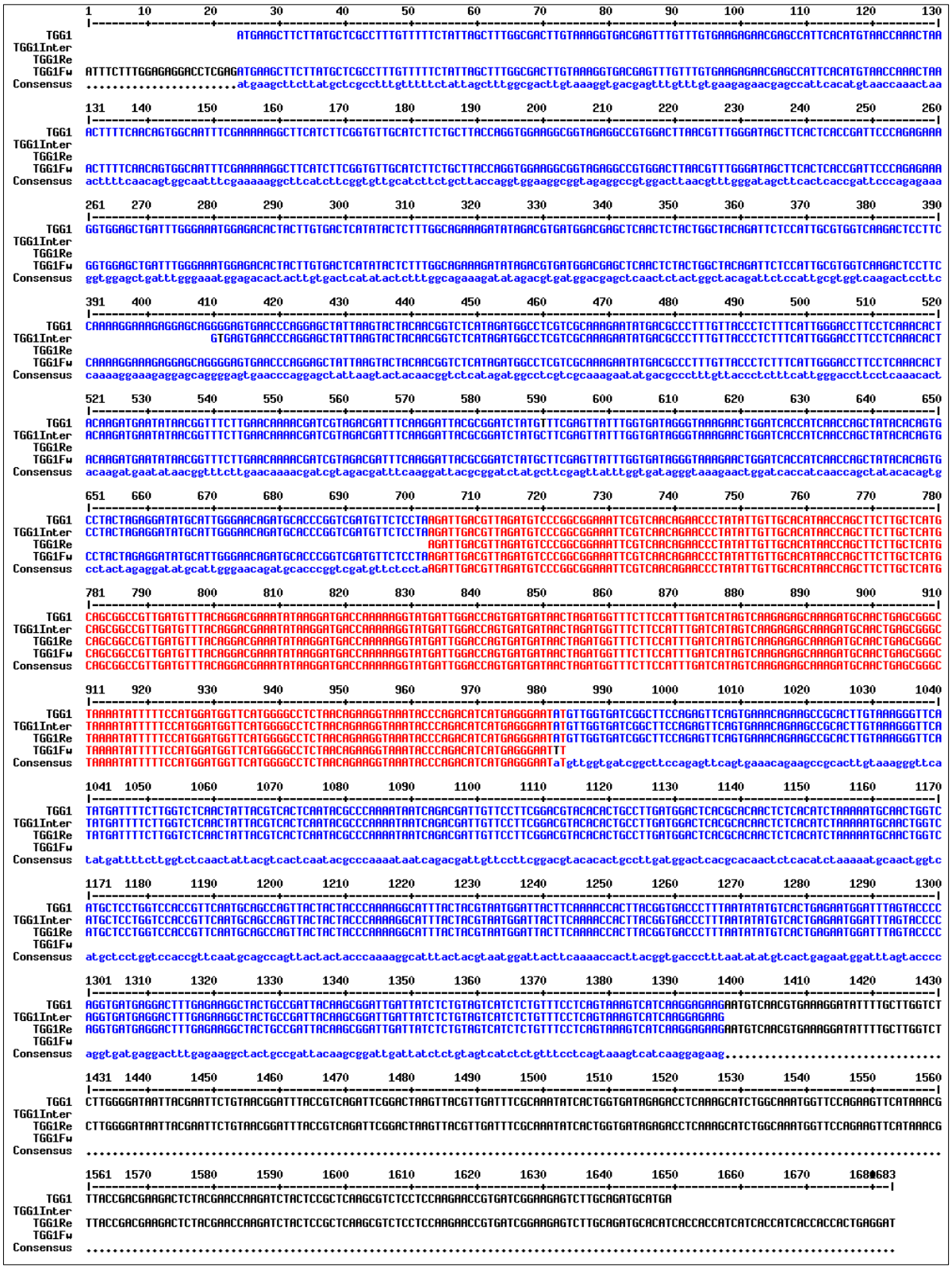

Fig. 7.2: Comparison of nucleotide sequence between TGG1 from Genbank and TGG1-*His* tag cloned in pRT101. TGG1: nucleotide sequence from data bank; TGG1 Int: sequence by using internal primer; TGG1Re: sequence by using reverse primer; TGG1Re: sequence by using forward primer.

7.3 Constructs for transfer of the glucosinolate biosynthesis pathway to plants

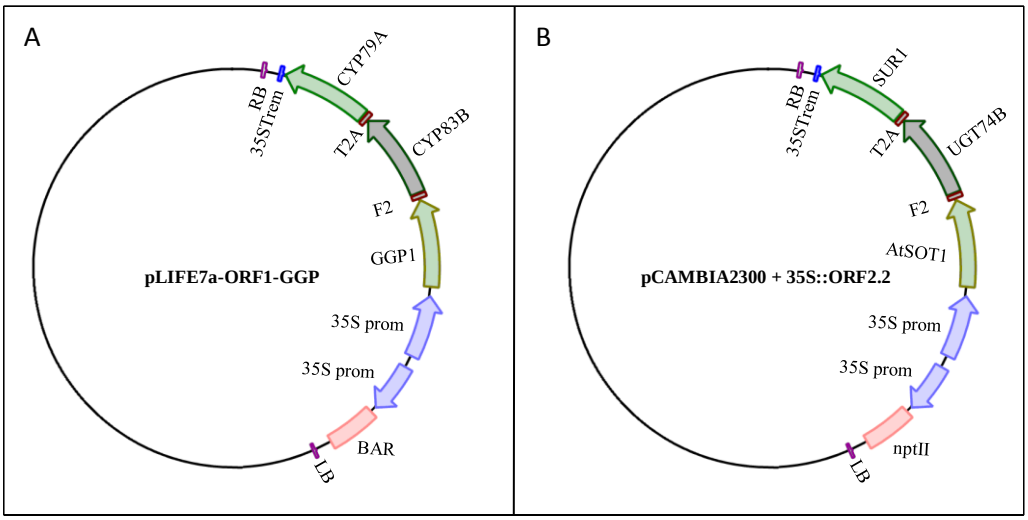

Fig. 7.3: Constructs for expression of genes for benzylglucosinolate biosynthesis. A: pLIFE7a-ORF1-GGP; B: pCAMBIA2300 + 35S::ORF2.2.

7.4 Preparation of protoplasts

Protoplasts are formed by enzymatic degradation of the cell wall. The enzyme mixtures to be used depend on the composition of the cell walls of the tissue used for protoplast isolation. Protoplasts from carrot suspension cultures were isolated according to Zhang et al. (2011), with modification. 10-day-old suspension culture cells were transferred into 0.6 M mannitol for 1 h in the dark at 23°C with gentle shaking at 60 rpm. The supernatant obtained by centrifugation at 1,000 rpm for 3 min was discarded. Each 1 g of cells was incubated overnight in 10 ml of enzyme solution (see below) in the dark with gentle shaking at 60 rpm. After the enzymatic digestion, an equal volume of W5 solution (see below) was added, followed by vigorous shaking by hand for 10 sec. The released protoplasts were separated from undigested tissue by filtration through a 100 and 70 µm nylon membrane (Omnilab, Germany). The pellet was collected by centrifugation at 1,500 rpm for 3 min. After washing twice with W5 solution, the pellet was re-suspended in fourfold volume MMG solution (see below). The protoplasts were viewed and photographed under a Zeiss microscope which was documented with the software dhs-Bilddatenbank® (dhs Dietermann & Heuser Solution GmbH,Germany) (Fig. 7.4).

Enzyme solution	1.5 % Cellulase RS 0.75 % Macerozyme R-10 0.6 M Mannitol 10 mM MES at pH 5,7 10 mM $CaCl_2$ 0.1 % BSA, sterile filtration	W5 solution	154 mM NaCl 125 mM $CaCl_2$ 5 mM KCl 2 mM MES at pH 5.7, sterile filtration

MMG solution	0.4 M Mannitol 15 mM $MgCl_2$ 4 mM MES at pH 5.7, sterile filtration

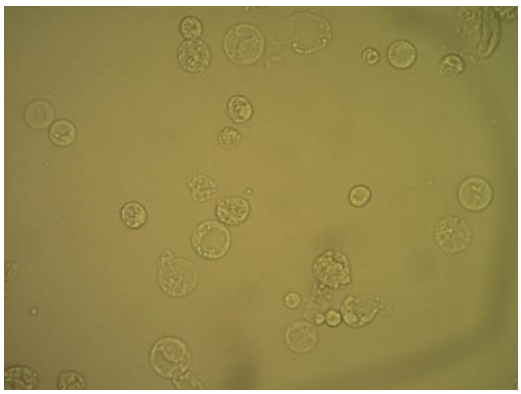

Fig. 7.4: Freshly isolated protoplasts from carrot callus suspension culture. Protoplasts were isolated according to Zhang et al., (2011), with modification. The isolated protoplasts are spherical because they are unrestricted by the cell wall. The number of protoplasts was limited and this may be due to the dilution of protoplast cells with the re-suspended MMG solution.

Viability of the protoplast is defined as the ability of isolated protoplasts to continue to grow in culture to replace its lost cell wall and to form suspension cells, callus or plantlets. Protoplasts viability was determined by the Mosmann assay that employed the mitochondrial-dependent reduction of MTT (3-(4-5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) to formazan. For the MTT assay, freshly isolated protoplasts (500 µl) were mixed with 50 µl of 0.2 mg/l MTT solution (Acros) and incubated at 30°C for 2 h. The suspension was then mixed with 500 µl of isopropanol containing 0.04 N HCl to solubilize the formazan. The mixture was vigorously vortexed to extract MTT-formazan from cells and then centrifuged at 1500 × g for 5 min. The absorbance of the supernatant was measured at 570 nm (Fig. 7.5). In MTT assay, when the number of viable cells increases, results in increasing in the amount of

MTT formazan formation which leads to increase in the absorbance. The problem that faced during this test was that the purple color of formazan formation not only observed in the isolated protoplast but also in the negative control, although the correlation of formazan absorbance was increased linearly with increased the amount of protoplast.

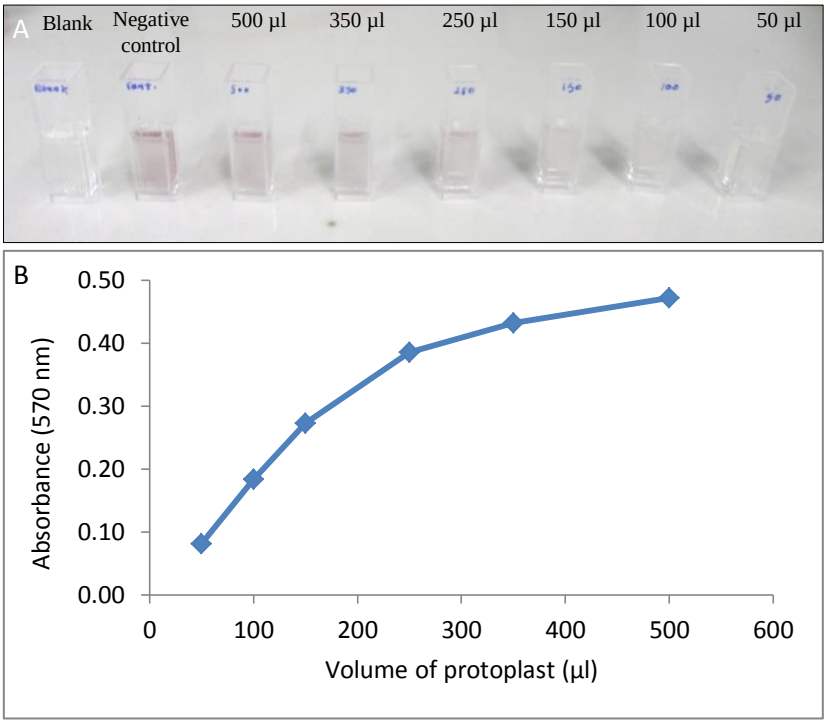

Fig. 7.5: MTT assay for protoplasts isolated from carrot callus suspension culture. A: Density of purple color with different protoplast volumes compared to the negative control (500 µl protoplast subjected to 95°C for 10 min); B: Relationship between the volume of protoplasts and production of MTT-formazan.

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